

**RECEPTOR REGULATION OF VOLUME-SENSITIVE OSMOLYTE  
EFFLUX FROM NEURAL CELLS**

**by**

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## LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine mono-phosphate
ANOVA	Analysis of variance
ATP	adenosine triphosphate
AVP	arginine vasopressin
BAPTA-AM	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester
BIM	bisindolylmaleimide
$[Ca^{2+}]_i$	cytoplasmic calcium
$Ca^{2+}$	calcium
CaMKII	calcium calmodulin-dependent protein kinase II
CD	methyl- $\beta$ -cyclodextrin
Cl <sup>-</sup>	chloride
CIC	family of chloride channels or transporters
CNS	central nervous system
DAG	diacyl glycerol
DCPIB	4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1 <i>H</i> - inden-5-yl)oxy]butanoic acid
DDF	1,9-dideoxyforskolin
DIDS	4,4'diisothiocyanatostilbene-2,2'-disulfonic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
EGTA	ethylenediycol- <i>bis</i> (2-aminoethyl)-N,N,N',N'-tetraacetic acid
Epicholesterol	5-cholesten-3 $\beta$ -ol
FAK	focal adhesion kinase
GDP	guanine diphosphate

GEF	guanine exchange factors
GPCR	G-protein coupled receptor
GTP	guanine triphosphate
HEPES	N-[2 hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IP <sub>3</sub>	inositol 1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
K <sup>+</sup>	potassium
KN-93	2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-(methylbenzylamine)
LPA	lysophosphatidic acid
mAChR	muscarinic acetylcholinergic receptor
MAPK	mitogen activated protein kinase
MLCK	myosin light chain kinase
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
Oxo-M	Oxotremorine M
PAR	proteinase or protease -activated receptor
PI3K	phosphatidylinositol-3'kinase
PIP2	phosphatidyl inositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PPACK	D-Phe-Pro-Arg Chloromethyl Ketone
PTK	phosphotyrosine kinase
PTX	pertussis toxin
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
S1P	sphingosine-1-phosphate
SITS	4-acetamido-4'-isothiocyanatostilbene-2, 2'-disulfonic acid
TCA	trichloroacetic acid
VSOAC	volume-sensitive organic osmolyte and anion channel

## ABSTRACT

Cell volume regulation is a homeostatic imperative in the brain due to the restrictions of the skull. To counteract an osmolarity disturbance and restore normal cell volume, neural cells initiate volume regulatory mechanism by modifying the concentration of their intracellular inorganic and organic osmolytes. This dissertation illustrates the important ability of GPCRs to potentiate osmolyte efflux in response to hypo-osmotic stress.

I discovered that the addition of sub-nanomolar concentrations of thrombin, mediated via the activation of PAR-1 receptor, can robustly enhance the release of the organic osmolyte taurine, through the volume sensitive organic osmolyte and anion channel (VSOAC) from neuro-tumor cells in response to hypo-osmotic stress. Biochemical and pharmacological studies demonstrated that both intracellular  $\text{Ca}^{2+}$  and PKC are required for receptor-mediated, but not basal (swelling-induced), taurine release. The results indicated that activation of PAR-1 receptors by thrombin lowers the threshold osmolarity or “set-point” for osmolyte efflux, thereby facilitating the ability of cells to respond to small reductions in osmolarity. Receptor activation (PAR-1 and mAChR) also enhanced the release of the inorganic osmolyte,  $\text{Cl}^-$  ( $^{125}\text{I}^-$  used as a tracer). The kinetics and magnitude of  $^{125}\text{I}^-$  release were greater than those of taurine. However, both osmolytes showed similar pharmacological profiles in response to

VSOAC inhibitors. Although receptor stimulated-aurine efflux was inhibited by depletion of intracellular  $\text{Ca}^{2+}$  and/or inhibition of PKC,  $^{125}\text{I}^-$  efflux was either unaffected or less dependent upon these two parameters. This differential regulation of the osmolyte release suggests either the presence of separate but pharmacologically similar, efflux channels or, receptor-specific activation of distinct signal transduction pathways that differentially contribute to the release of taurine and  $^{125}\text{I}^-$ , both of which are released through a common membrane channel.

I also demonstrated that cholesterol depletion with methyl- $\beta$ -cyclodextrin synergistically potentiated receptor-mediated taurine release. This stimulatory effect was not due to disruption of lipid rafts or changes in receptor signaling. Experiments with cholesterol analogs provided evidence that the potentiation by cholesterol depletion resulted from changes in the biophysical properties (fluidity) of the membrane. Together, the findings described in this thesis provide new insight into mechanisms involved in the GPCR-regulation of osmolyte efflux following hypo-osmotic stress in neural cells.

## **CHAPTER 1**

### **INTRODUCTION**

#### **Evolutionary Conservation of Cell Volume Regulation**

Cell volume regulation is a homeostatic trait conserved in essentially all species throughout evolution. Bacteria readily change their intracellular osmolarity in response to alterations in their environment. This ability makes them able to survive in a wide variety of habitats (Csonka, 1989). Due to the presence of a rigid cell wall, fungi, algae and plants are less susceptible to small osmolarity changes in their environment. This cell wall, therefore, prevents cell swelling but not shrinkage. Normally these cells maintain their shape by the osmotic balance between the cytosol and their environment (turgor) (Yoshida et al., 1997; Johansson et al., 2000). The osmolarity of marine invertebrates is matched to the concentration of surrounding sea-water by adjusting the intracellular concentration of ions or organic osmolytes. In other multicellular organisms such as fish and mammals, cells are in contact with interstitial fluid and each individual cell contains a variety of plasma membrane transporters and channels to maintain homeostatic conditions. Animal cells have flexible but fragile membranes which require osmotic volume regulation to prevent them from collapse by shrinkage or lysis by swelling (Strange, 2004).

Although the process of cell volume regulation has been preserved throughout evolution, the cellular mechanisms and the osmolyte profiles involved vary considerably. Protozoa achieve osmoregulation predominantly through efflux of amino acids, of which alanine is quantitatively the most important. In contrast, efflux of inorganic anions is most important in mammalian cells, and the most abundant amino acid involved in cell volume regulation is usually taurine (Krogh, 1939; Yancey, 1982). The cellular response to osmotic stress ensures that the content of water inside the cell is maintained within a range that is compatible with biologic function.

Approximately 60% of body weight in man consists of water with two-thirds being present inside cells and most of the remaining water comprising the interstitial fluid outside cells that forms the extracellular environment. In mammals, protection of cells against high osmolarities is especially required in the kidney, where osmolarities up to 1000 mOsM can develop during antidiuresis (Hall et al., 1996; Schliess and Haussinger, 2002). In the brain, this homeostasis is absolutely critical due to the spatial constraints imposed by the skull. Expansion of intracranial tissue can cause constriction of small blood vessels, episodes of anoxia, ischemia, excitotoxicity and ultimately cell mortality.

### ***Osmoregulation***

The movement of water between aqueous solutions that are separated by a semi-permeable membrane is driven by differences in the concentration of solute particles (also referred to as osmolytes) on either side of the membrane,

with water flowing down its concentration gradient from the solution with a lower osmolyte concentration into the solution with a higher concentration. This process, referred to as osmosis, is of fundamental and universal significance to all biologic organisms as they utilize a semi-permeable membrane to separate the intracellular milieu from the extracellular environment. The osmotic flow of water occurs by passive diffusion across the lipid bilayer, however some cells may possess water-permeable protein channels called aquaporins to accelerate this water transport (Agre and Kozono, 2003; King et al., 2004).

A multitude of factors challenge the constancy of cell volume. These include alterations in extracellular osmolarity, changes associated with cellular processes such as synthesis and breakdown of macromolecules, accumulation of metabolites, exocytosis and dissipation of ion gradients. A challenge to cell volume can only be accommodated if osmotic equilibrium across the cell membrane is maintained by cell volume regulatory mechanisms. Cell volume regulation is linked to many aspects of cell function, including glucose metabolism, pH regulation, cell excitability, protein function and cell survival. It is a universal feature for cell growth and division of all cells.

There are two types of volume changes that activate osmoregulatory mechanisms; anisosmotic volume changes are induced by alterations in extracellular osmolarity, while isosmotic volume changes are brought about by alterations in intracellular solute content (Kimelberg, 1995). The focus of this thesis is on how cells respond to changes in their extracellular environment.



## **Cell Volume Adaptation**

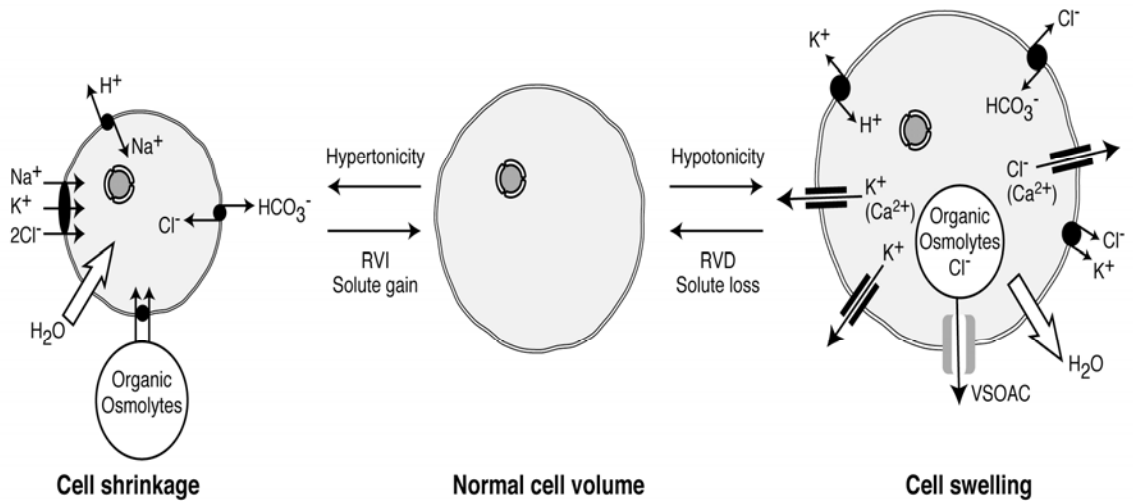
Under steady state conditions, the osmotic concentrations of cytoplasmic and extracellular fluid are in equilibrium. In the face of external or internal challenges, cells alter the concentration of osmolytes in order to reduce the difference in intracellular versus extracellular concentration, eliminating any change in intracellular water concentration and therefore a concomitant change in cell volume occurs. This flux of water either in or out of the cell results in cell swelling or cell shrinkage, respectively. If the cell volume in response to osmotic stress is not corrected, it may cause cell damage and loss of function. For example, cell swelling can inhibit glycolysis, stimulate glycine oxidation and glutamine breakdown and form  $\text{NH}_4$  and urea from amino acids while also decreasing the mRNA expression of principal enzymes involved in gluconeogenesis (Haussinger et al., 1994a). Therefore all cells have a mechanism for restoration of cell volume that is accomplished by the fast activation of potassium ( $\text{K}^+$ ), sodium ( $\text{Na}^+$ ) and chloride ( $\text{Cl}^-$ ) channels, exchangers and cotransporters. Cell swelling, due to reduction in extracellular tonicity triggers Regulatory Volume Decrease (RVD) and cell shrinkage, due to high extracellular tonicity results in Regulatory Volume Increase (RVI) (Figure 1.1).

### ***Regulatory Volume Decrease (RVD)***

When cells are exposed to a decrease in osmolarity (hypotonic environment), there is an increase in cell volume or hypo-osmotic cell swelling.

This volume increase is transient and is followed by a regulatory volume decrease (RVD). RVD is an active process that involves the release of cations such as  $K^+$  and anions such as  $Cl^-$  via activation of separate  $K^+$  and  $Cl^-$  channels or by activation of the K-Cl cotransporter. In a few cell types,  $K^+$  and  $Cl^-$  efflux is mediated by activation of  $K^+/H^+$  and  $Cl^-/HCO_3^-$  exchangers. As these electrolytes are released from the cell they bring with them obligated water in part by specialized water channels called aquaporins (For review, see Verkman and Mitra, 2000, Agre et al., 2002). This net flux helps the cell return to its original volume and establish a new osmotic equilibrium.

Finally, osmotic cell swelling also induces a rapid release of organic osmolytes such as taurine, alanine, inositol through a yet unidentified volume sensitive organic osmolyte and anion channel (VSOAC) that primarily transports  $Cl^-$ . Since loss of inorganic osmolytes such as  $K^+$  and  $Cl^-$  can be potentially harmful on a long-term basis, release of these electrolytes reverses with time, whereas the release of organic osmolytes is more sustained (Olson, 1999). The efflux pattern of organic osmolytes also closely parallels the time course of the change in cell volume, in contrast to  $Cl^-$  and  $K^+$  fluxes which are faster ( $Cl^-$ ) or slower ( $K^+$ ) than the change in cell volume (Pasantés-Morales, 1998). Downregulation of the mechanisms controlling synthesis and uptake of organic osmolytes also contributes to the loss of the solutes. In many cases, exposure of cells to hypotonic media has been shown to cause a regulation of the rates of both efflux and influx for a range of solutes. The net flux of organic osmolytes is usually out of the cell (Lambert, 2004).



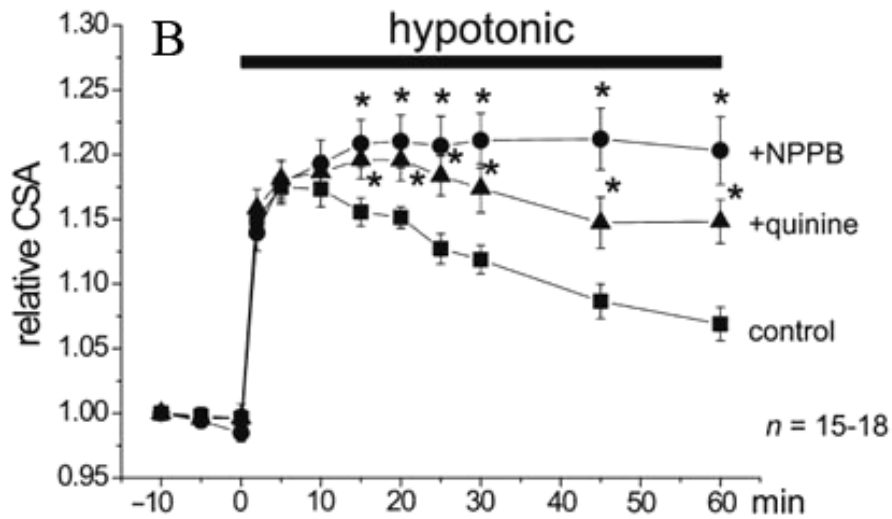
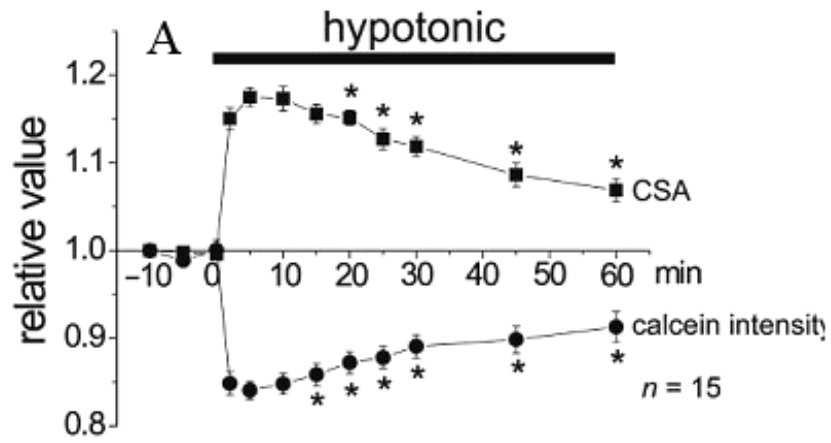
**Figure 1.1. Cell volume adaptation**

Under conditions of hyperosmotic-induced cell shrinkage, NaCl influx is effected via the  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  antiporters or by operation of the  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  symporter. Organic osmolytes (e.g., taurine or inositol) are also transported into the cell via  $\text{Na}^+$ -dependent carriers, which increase in number. During hypotonic-induced cell swelling, KCl is extruded via separate  $\text{K}^+$  and  $\text{Cl}^-$  channels (either  $\text{Ca}^{2+}$ -activated or  $\text{Ca}^{2+}$ -independent), a  $\text{K}^+/\text{Cl}^-$  symporter and  $\text{K}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  antiporters. The efflux of organic osmolytes occurs via a channel that primarily transports  $\text{Cl}^-$  ions (VSOAC). (B. Med Illustrator)

The time or rate necessary to regain cell volume varies among different cell types. In cultured astrocytes, RVD occurs rapidly with a 70-80% recovery reached within a few minutes (Pasantes-Morales, 1996). In vivo, a decrement in brain sodium and chloride content can be measured within 30 minutes, whereas a decrement in potassium is slower, measurable only at 3 hours after the onset of cell swelling (Melton et al., 1987; Berl, 1990; Figure 1.2). The focus of my thesis will be on the process of RVD.

### ***Regulatory Volume Increase (RVI)***

Exposure of a cell to an increase in osmolarity (hypertonic environment) results in the osmotic efflux of water and a reduction in cell volume (i.e., cell shrinkage). Cell shrinkage in response to a hyperosmotic challenge stimulates a net uptake of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  ions and osmotically obligated water leading to a restoration of "normal" cell volume, a process known as regulatory volume increase (RVI) (Cserr et al., 1991). RVI in a majority of cells involves the concomitant activation of  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchanger as well as the  $\text{Na}^+ \text{K}^+$  ATPase and  $\text{Na}^+/\text{K}^+ / 2\text{Cl}^-$  (NKCC) cotransporter (O'Neill, 1999; Lang et al., 1998c). Upregulation of NKCC cotransporter may also be essential during a long-term hyperosmotic challenge (Bildin et al., 2000). Adaptation to hyperosmolarity is a relatively slow process and may take several hours since accumulation of organic osmolytes also involves an increased gene transcription, elevated mRNA levels and increased synthesis of functional transporters (Yancey et al., 1982).



**Figure 1.2. Regulatory volume decrease (RVD) after hypotonic swelling in cortical neurons.** (A) Time course of cell size change during exposure to 63.5% hypotonic solution monitored by both cross-sectional area (CSA) and the fluorescence intensity of intracellular calcein. (B) Effects of Cl<sup>-</sup> channel blocker, NPPB and K channel blocker, quinine on RVD (Adapted from Inoue et al., 2005).

In the event that biochemical homeostasis is not restored, the resulting disequilibrium gives rise to a wide spectrum of deleterious effects on cell function and ultimately results in apoptotic cell death.

### ***Experimental measurement of cell volume***

A variety of methods are available for measuring absolute and relative cell volume changes under experimental conditions including electronic sizing by Coulter-type impedance techniques, light scattering, ion-sensitive electrode and radioactive tracer methods. Microscopy methods such as calcein-AM fluorescence microscopy can measure the intensity of fluorescent probes trapped within the cell and reliably track swelling and shrinkage-induced volume perturbations as long as the probes are not sensitive to cellular parameters such as pH or calcium etc (Parkerson and Sontheimer, 2003, 2004; Inoue et al., 2005).

### **Osmolyte Release: Inorganic and Organic Osmolytes**

#### ***Potassium conductance***

The intracellular and extracellular concentrations of  $K^+$  in cells are 140 mM and 5 mM respectively. Despite the identification of a large number of general  $K^+$  channels, very little is known about the  $K^+$  channels which respond to hypo-osmotic swelling and their mechanism of regulation. Swelling activates at least two different types of  $K^+$  channels. In some cells, mainly epithelial cells, volume-sensitive  $K^+$  channels (VSKC) are calcium-dependent and large conductance

(100 – 200 pS) channels. In other cell types,  $K^+$  channels are small channels with conductance of 20-30 pS, the majority of which are calcium-independent (For review, see Pasantes-Morales and Morales Mulia, 2000). The calcium-dependent  $K^+$  channels that are activated only by osmolarity show a pharmacological profile characterized by insensitivity to typical  $K^+$  channel blockers such as tetraethylammonium (TEA), quinidine, 4-aminopyridine and barium but sensitivity to clofilium (Ordaz et al., 2004b). Therefore depending on the cell type examined, volume regulatory  $K^+$  channels include Kv 1.3, Kv 1.5 (voltage-sensitive K channels), the mini K channels and members of the 4M two-pore family of channels (Deutsch and Chen, 1993; Pasantes-Morales and Morales-Mulia, 2000; Niemeyer et al., 2001a, b; Barfield et al., 2005). These  $K^+$  transport mechanisms activated by cell swelling are now also known to play an important role in initiating apoptosis (Trimarchi et al., 2002). Electroneutral  $K^+ Cl^-$  (KCC) symport activity is also known to be involved in RVD (Wehner et al., 2003; Shen et al., 2004).

The activation of  $K^+$  channels is effective for volume regulatory ion release only if the anion channels are operating in parallel, since prolonged release of  $K^+$  could lead to hyperexcitability of the cells without a significant regulatory volume decrease (Lang et al., 1998).

### ***Chloride conductance***

Chloride concentration inside cultured cells can reach ~40 mM and therefore chloride anion channels are found in nearly every cell type known

(Kettenmann, 1987). Cl<sup>-</sup> channels play a crucial role in controlling the ionic composition of the cytoplasm and the volume of cells. In addition to their pivotal role in cell volume regulation, they participate in a variety of functions including transport across epithelia, acidification of intracellular organelles, stabilization of cell membrane potential and glioma cell migration (Nilius and Droogmans, 2003; McFerrin and Sontheimer, 2006). These functions are performed in a close interplay with various ion transporters, including pumps, cotransporters and other ion channels (Jentsch et al., 2002).

### ***Volume Sensitive Organic osmolyte and Anion Channel (VSOAC)***

A robust chloride conductance is observed by whole-cell electrophysiology when a wide variety of cells are exposed to hypo-osmolar solutions (decrease in osmolarity). The channel exhibits the following characteristics (a) outward rectification with an intermediate unitary conductance of 40-78 pS (b) time-dependent inactivation at positive membrane potential above 60 mV and recovery from inactivation at negative potentials (c) a permeability sequence of SCN<sup>-</sup> > I<sup>-</sup> > Br<sup>-</sup> > Cl<sup>-</sup> > HCO<sub>3</sub><sup>-</sup> >> taurine > gluconate > aspartate, corresponding to the Eisenmann's sequence I and (d) requirement for ATP, but not its hydrolysis for its activation, since non-hydrolyzable analogs of ATP, ADP or GTP can be substituted (Jackson et al., 1994; Strange et al., 1996) (e) sensitivity to chloride channel blockers such as disulfonic stilbenes DIDS, SITS, as well as NPPB, DDF and niflumic acid (Nilius et al., 1996; Voets et al., 1997; Nilius and Droogmans 2003; Suzuki et al., 2006). These chloride channel blockers are



however, rather non-specific and have low potency. DCPIB, a derivative of ethyrcinic acid (Decher et al., 2001; Best et al., 2004) is so far considered to be the most specific agent yet identified for inhibiting swelling-activated chloride channels (Abdullaev et al., 2006).

The current through this channel closely correlates with changes in cell volume and has been labeled as volume- or swelling-activated  $\text{Cl}^-$  current, or  $I_{\text{Cl}^-}$  swell. This ubiquitously expressed channel has also been referred to as volume-regulated anion channel (VRAC), volume expansion sensing sensor (VSOR) or volume sensitive organic osmolyte and anion channel (VSOAC) (Jackson et al., 1994; Okada, 1997). The permeability ratio ( $P_x/P_{\text{Cl}^-}$ ) for the different osmolytes range from  $\sim 0.1$  for glutamate,  $\sim 0.2$  for taurine to  $\sim 0.6$  for the small amino acid, glycine. The pore diameter of VSOAC lies between 5.4 and 8 Å ( $\sim 1.1$  nm) (Jackson and Strange, 1995; Nilius et al., 1997). This large diameter of the channel may explain the range of permeating compounds allowing  $\text{Cl}^-$  anion (3.6 Å; Franciolini and Nonner, 1987) as well as organic osmolytes such as glycine and inositol (4.4-5.9 Å) to readily permeate (Strange and Jackson, 1995). There is also some evidence suggesting that VSOAC itself can be permeated by water and its closure would reduce the overall water permeability (Nilius, 2004). VSOAC has also been implicated in setting the membrane potential, in modulating salt and fluid secretion as well as cell cycle progression, cell proliferation, angiogenesis, mechanosensitivity of various cell types and also in apoptosis (Okada and Maeno, 2001; Nilius and Droogmans, 2003).

Although the VSOAC has been pharmacologically and electrophysiologically characterized, its molecular identity is still not known and the gene or genes encoding the channel mediating the swelling activated  $\text{Cl}^-$  current have not yet been identified. Reasons for this include the ubiquity of the VSOAC and the absence of a specific high-affinity ligand. There have been certain candidates for the VSOAC and these include:

- members of the CIC family (Duan et al., 1997; Wang et al., 2000; Jin et al., 2003)
- phospholemman (Moorman et al., 1998; Morales-Mulia et al., 2000; Moran et al., 2001)
- P-glycoprotein (Multi-drug resistant protein (MDR); Hardy et al., 1995; Valverde et al., 1996)
- $\text{ICl}_n$  (Krapivinsky et al., 1994; Musch et al., 1998)
- maxi anion channel (see Nilius et al., 1997 for review)

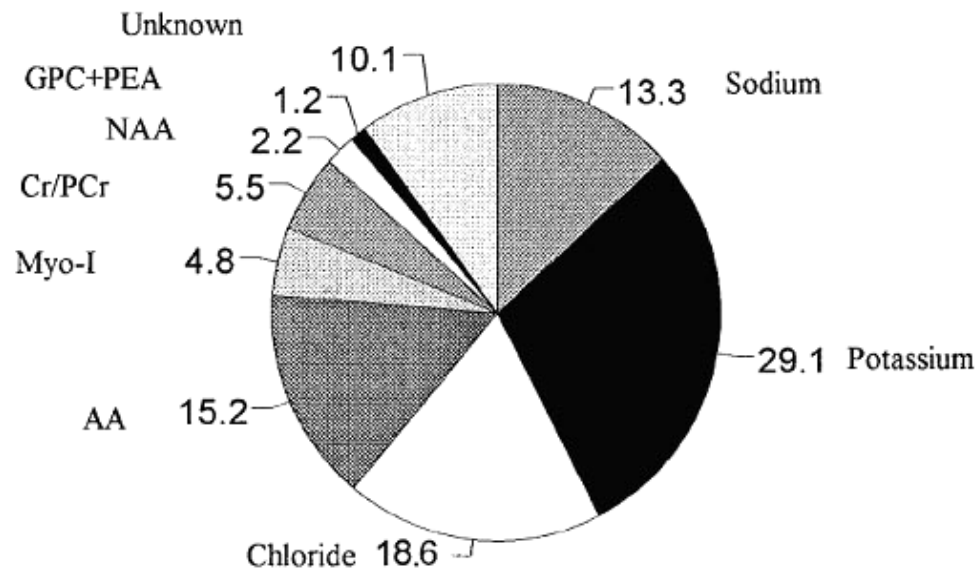
However some of the electrophysiological and pharmacological properties of these  $\text{Cl}^-$  channels are at variance with VSOAC and organic osmolyte efflux and none of them fulfill all the requirements to function as a universal osmo-sensitive  $\text{Cl}^-$  channel (Vanoye et al., 1997; Pasantes-Morales et al., 1997; Moran et al., 1997; Li et al., 2000; Weylandt et al., 2001).

### ***Organic Osmolytes***

Organic osmolytes are found in high concentration (10 to 100 mM) in the cytosol of all organisms from bacteria to humans. Although inorganic ions are the

quantitatively major osmolytes, contributing 62-70% of total osmolyte content, they are insufficient to compensate for the entire loss of water (Lang et al., 1998b). Therefore, organic osmolytes (23-29%) are important to be considered and can be grouped into three classes (1) polyols (*glycerol, sorbitol, myo-inositol*) (2) amino acid and derivatives (*taurine, glutamate, alanine, proline*) and (3) methylamines (*betaine, glycerophosphorylcholine*) (Pasantes-Morales et al., 2002a; Figure 1.3). Unlike inorganic ions that can interfere with structure and function of proteins and nucleic acids, most organic osmolytes, even if accumulated at high concentrations, are neutral (either zwitterionic or lacking charge at physiological pH), non-perturbing “compatible” solutes without any deleterious effects. Changes in their concentration do not significantly affect membrane potential, enzyme activities or ionic strength of the cytoplasm, which is of particular importance especially for excitable cells such as neurons. Many of these organic osmolytes, are in turn “counteracting” and have cytoprotective properties such as anti-oxidation and stabilization of proteins (Hoffman and Lambert, 1983; Lang et al., 1998a).

Different tissues and organs of the body have different distributions of organic osmolytes, the concentration of which varies through the developmental stages of growth and differentiation (Miller et al., 2000). Whether it's the kidney enriched in sorbitol, myo-inositol, glycerophosphorylcholine and betaine or the heart, brain and eye lens with its myo-inositol and taurine concentration, more and more studies suggest the importance of these organic osmolytes in mammals (Schaffer et al., 2000b; Yancey, 2005).

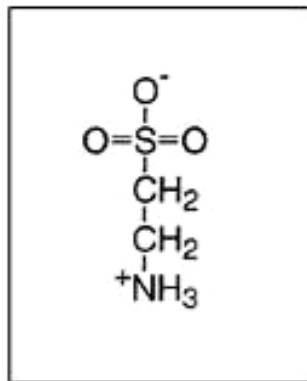


**Figure 1.3. Relative contribution of various inorganic and organic osmolytes to RVD in the brain.** Inorganic and organic osmolytes involved in the control of brain water content during chronic hyponatremia. GPC: glycerophosphorylcholine, PEA: phosphatidyl ethanolamine, NAA: N-acetyl aspartate, Cr/PCr: creatine/phosphocreatine; Myo-I: myo-inositol, AA: amino acids (Adapted from Pasantes-morales et al., 2002a).

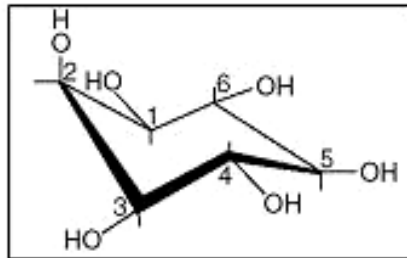
In the central nervous system (CNS), the three major organic osmolytes are taurine, glutamate and myo-inositol. These osmolytes differ in their sizes and do not have similar structures (Figure 1.4; Jackson and Strange, 1993; McManus et al., 1995; Nilius et al., 1997). Generation of these osmolytes is a relatively slow process requiring months to days and are accumulated by cells in three ways: increased synthesis, decreased degradation and by transport across the plasma membrane via Na<sup>+</sup> dependent transporters. Although a high concentration of glutamate is involved in ischemic brain injury and spreading depression, the release of glutamate as an osmolyte through VSOAC is now suggested to play an important role in synaptic communication and astrocyte-neuron signaling (Mulligan and MacVicar, 2006).

### ***Taurine, the ideal osmolyte***

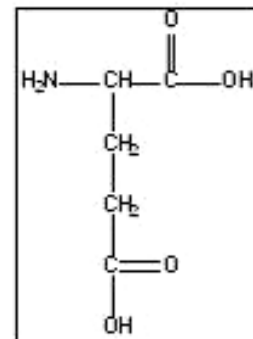
Taurine (2-aminoethanesulphonic acid) was isolated from ox bile in 1827 where it is found in high concentrations (Jacobsen and Smith, 1968). Chemically, taurine is distinct from other amino acids in that it is not chiral, and like glycine does not polarize light. It occurs in the body as a free molecule and is never incorporated into muscle proteins. The high water solubility, very limited permeation across cell membranes and poor ability to chelate cations make the non-cytotoxic taurine well suited as osmolyte for adjustment of the intra-cellular osmotic pressure and cell volume after osmotic perturbation (Huxtable, 2000).



Taurine



Myo-Inositol



Glutamate

**Figure 1.4. Structure of three quantitatively major organic osmolytes in the central nervous system.**

Although taurine has been reported to form the carboxylic terminal of some low molecular weight brain synaptic peptides (Marnela et al., 1985) and to be incorporated into membrane lipids (Kaya and Sano, 1991), most of taurine remains free as a zwitterion in the body fluids. Taurine is not considered an essential amino acid, because most carnivores and omnivores can synthesize it from a combination of two natural amino acids, cysteine and methionine, with the help of vitamin B6. However new born mammals including cats and certain monkeys have little or no capacity for de novo taurine biosynthesis and therefore rely on dietary supply of taurine (Gaull, 1989). Taurine has been claimed to be of benefit in the treatment of congestive heart failure and affords protection against arrhythmogenesis, however the exact mechanism for this is still not known (Schaffer et al., 2000a; Gupta et al., 2005). Lack of sufficient taurine is also considered to be associated with cardiomyopathy, retinal degeneration and growth retardation (Lombardini, 1991; Aerts et al., 2002). Nevertheless the main purported functions for taurine are 1) bile acid conjugation 2) detoxification (Birdsall, 1998) 3) osmoregulation (Hussy et al., 2000) 4) modulation of intracellular calcium (Xu et al., 2006b).

Although taurine has been thought of as an inhibitory neurotransmitter since it exhibits effects as a weak agonist at GABA<sub>A</sub> and glycine receptors, it is not a bona fide neurotransmitter (Hussy et al., 1997). Although taurine does not scavenge reactive oxygen species (ROS), it can directly bind to HOCl to form stable N-chlorotaurine (Aruoma et al., 1988; Schaffer et al., 2003). Hypotaurine, a taurine derivative on the other hand is a strong antioxidant and is known to

occur at significant levels in mammalian reproductive fluids and in marine animals living in sulfide-laden waters (Setchell et al., 1993). Taurine influx against a steep concentration gradient is accomplished by a taurine transporter (TauT) that is dependent on  $\text{Na}^+$ ,  $\text{Cl}^-$  and pH and is driven by the transmembrane electrochemical gradient (Lambert and Hoffman, 1993; Han et al., 2006).

Taurine is the most intensively studied compatible solute in counteracting RVD and often used as a representative of the organic osmolyte pathway. Large quantities of taurine can be lost from cells without altering the resting membrane potential or modifying cell function. The sulfonic amino acid taurine has been conserved strongly from amoebae to mammals and is present at high concentration (5-50 mM) in neuronal cells (Lehman and Hansson, 1987; Huxtable, 1989; Olson et al., 2003) and mammalian heart (Miller et al., 2000). In fact, it has been demonstrated that the volume regulatory capability of cultured astrocytes and hippocampal slice cultures varies with intracellular taurine concentration, with diminished effects in taurine-deficient media (Moran et al., 1994, 1996a; Kreisman and Olson, 2003). Taurine concentrations have been demonstrated to be reduced from 53 to 7 mM within 40 min after transfer to hypotonic medium (50% reduction in osmolarity) in Ehrlich tumor cells (Hoffmann and Lambert, 1983) and an equivalent loss of taurine in response to cell swelling in vivo has also been demonstrated by intracranial microdialysis (Wade et al., 1988). Taurine is the osmolyte which is most sensitive to osmotic perturbation, with the lowest release threshold and largest amount released in most mammalian cells (Pasantés-Morales et al., 2000b). Taurine is not concentrated in



secretory granules but is equally distributed in the cytoplasm and in the nucleus (Bustamante et al., 2001). At normal physiological pH taurine is present predominantly as an electroneutral zwitterion and it is in this form that taurine is released from swollen cells (Guizouarn et al., 2000).

### **Pathways for Organic and Inorganic Osmolytes**

Arguments in support of the involvement of a  $\text{Cl}^-$  channel (VSOAC) as a major route for the volume-regulatory efflux of organic osmolytes comes mainly from electrophysiological studies showing that the swelling-activated  $\text{Cl}^-$  current, swelling-induced transport of organic osmolytes and RVD are all inhibited by a range of anion channel blockers (Strange et al., 1996). The pharmacological profile of organic osmolyte inhibition is similar to that of VSOAC, suggestive of a common pathway with  $\text{Cl}^-$  or of a close connection between the two pathways. The swelling-activated taurine efflux is not an exchange of cellular taurine for extracellular  $\text{Cl}^-$  via an anion exchange system (Jackson and Strange, 1993; Lambert and Hoffman, 1994;). It is  $\text{Na}^+$  independent, temperature-insensitive and not antagonized by guanidinoethane sulfonate (GES), an inhibitor of the taurine transporter. Taurine translocation and prompt release in response to swelling is passive, directed only by its concentration gradient (no energy dependent carrier) (Sanchez-Olea et al., 1991; Schousboe and Pasantes-Morales, 1992). Furthermore, the pathway mediating swelling-dependent efflux of taurine is shared by a variety of structurally unrelated organic molecules, does not appear to saturate with increasing concentrations of transported solute and is not

stereoselective. These characteristics of transport pathways are unlike those of typical membrane transporters. Instead they are those suggestive of a pore or channel (Kirk and Strange, 1998; Junankar and Kirk, 2000).

Although the evidence for a common osmosensitive permeability pathway for  $\text{Cl}^-$  and organic osmolytes is strong, there is a growing body of research data suggesting that the efflux profiles of different osmolytes under hypo-osmotic conditions may vary. There have been reports of differential activation and blockade of swelling activated taurine and  $\text{Cl}^-$  efflux in Ehrlich mouse ascites tumor cells and rat mammary tissue (Lambert and Hoffman, 1994; Shennan et al., 1994; Stutzin et al., 1999). Discrepancies between time-courses for inactivation and activation of  $^{125}\text{I}^-$  (used as a tracer for  $\text{Cl}^-$ ) and amino acid efflux have also been demonstrated in Chinese Hamster Ovary, NIH/3T3 and HeLa cells (Moran et al., 1997; Stutzin et al., 1999). The possibility of two or more isoforms of volume-sensitive taurine efflux pathways that are differentially regulated cannot be excluded (Mongin et al., 1999b). This has led to speculation regarding the possible existence of more than one isoform of VSOAC or separate permeation pathways for the osmolytes depending on the cell or tissue examined. Chapter 3 of my thesis investigates the similarities and differences of the release of taurine and  $^{125}\text{I}^-$  (tracer for  $\text{Cl}^-$ ) in neuroblastoma cells.

### **Physiological Significance of Cell Volume Regulation in the CNS**

Disturbances of body fluid osmolarity are common as clinical entities and can give rise to a diverse group of disease states and complications. Normal

plasma osmolarity is around 287 mOsM varying only about 1% from day to day. As little as a 2 mOsM increase in plasma osmolarity can activate thirst and arginine vasopressin (AVP) release from the posterior pituitary to offset the increased osmolarity (Mckinley and Johnson, 2004). This finely tuned homeostatic mechanism can be compromised in the face of acute osmotic stress from clinical intervention, accident or disease. The primary clinical manifestations of both hyper- and hypo-osmolar states are central nervous system dysfunction due to the restrictions of the skull. In the brain, even modest reductions in plasma osmolarity can raise the intracranial pressure and cause tissue damage. With hyperosmolar perturbations in plasma osmolarity, the brain passively shrinks as a result of secondary substantial cellular water loss. In hours to days, depending on the extracellular solutes, restoration of brain volume may be achieved if the solute is endogenous. This occurs largely by the generation of new, non-electrolyte intracellular solutes in the brain. On the other hand, in hypo-osmolar states, the brain expands its volume on the basis of passive water movement. In time, restoration of cell volume is achieved largely through loss of intracellular electrolytes as well as amino acids and myo-inositol (Lien et al., 1991; Pasantes-Morales et al., 2002b; Table 1.1). With time, these mechanisms appear to protect brain volume at the expense of the intracellular milieu. The resultant alteration of intracellular osmolyte composition may be largely responsible for the neurological symptoms observable in patients and experimental animals with such afflictions (Pollock and Arieff, 1980; Gullans and Verbalis, 1993).

The most common manifestation of a decrease in osmolarity leading to hypo-osmotic swelling is hyponatremia. Hyponatremia is a disorder of fluid-electrolyte balance (serum sodium concentration < 135 mM; normal being 145 mM) (Palm et al., 2006). The clinical prevalence of hyponatremia has been reported to be as high as 2.5% among hospitalized adult patients (Anderson et al., 1985; Bussman et al., 2001; Hoorn et al., 2006). A sustained decrease in plasma sodium concentration disrupts the osmotic balance across the blood-brain barrier, resulting in a rapid influx of water into the brain. This causes brain swelling and a cascade of severe neurological responses (Murray et al., 2004). Hyponatremia is associated with clinical conditions such as congestive heart failure, hepatic cirrhosis and renal failure, inappropriate secretion of the antidiuretic hormone (SIADH), glucocorticoid deficiency and use of thiazide diuretics (Haussinger et al., 1994b; Rovira et al., 2002; Pasantes-Morales et al., 2002b). Water overload that occurs in psychiatric disorders such as schizophrenia and psychogenic polydipsia can drop plasma osmolarity by 40 mOsm or more (Hayashi et al., 2005). In addition, neural swelling is also a major complication observed with stroke, traumatic brain injury, diabetes, excessive diarrhea or hepatic encephalopathy (Patel and Balk, 2007). This swelling, if uncorrected can be lethal as it can lead to compression of blood vessels, cerebral ischemia, infarct, excitotoxicity and eventually causing the displacement of the brain parenchyma through the foramen magnum (brain herniation) and respiratory arrest (Pasantes Morales et al., 2000). Paradoxically, if hyponatremia is corrected too rapidly (> 0.5mmol/L/h), high cerebral ion concentration and the

Osmolyte (nmol/kg DBW) <sup>a</sup>	Normonatremic	Hyponatremic (nmol/kg DBW)	Decrease	% dec
<b>Electrolytes</b>				
Sodium	279	250	29	10
Potassium	480	424	56	12
Chloride	152	118	34	22
All electrolytes	911	792	192	<b>21</b>
<b>Organic osmolytes</b>				
Glutamate	52.9	32.5	20.4	39
Glutamine	14.2	6.5	7.7	54
Taurine	13.8	2.1	11.7	<b>85</b>
GABA	1.7	0.9	0.8	47
Aspartate	2.2	1.7	0.5	23
N-acetylaspartate	7.5	5.9	1.6	21
Myo-inositol	16	5.3	10.7	<b>67</b>
Creatine	34.8	17.1	17.7	51
Phosphoethanolamine	1.2	0.8	0.4	33
GPC <sup>b</sup>	1.1	0.6	0.5	45
All organic osmolytes	145.4	73.4	72	<b>50</b>

<sup>a</sup>DBW: dry brain weight. Recalculated from (8,9). Data are from rats after 2–3 days of hyponatremia. <sup>b</sup>GPC: glycerophosphorylcholine.

**Table 1.1. Electrolytes and organic osmolytes in rat brain after chronic hyponatremia.** Immediate response to hyponatremia in brain involves K<sup>+</sup> and Cl<sup>-</sup> efflux, whereas sustained adaptation is carried out by organic osmolytes, particularly taurine. This has been confirmed in studies *in vitro* in astrocytes demonstrating how swelling-activated efflux of myo-inositol- and taurine persists for several hours after the hypotonic stimulus, in contrast to glutamate and K<sup>+</sup>, which remained unchanged. Note that the percent decrease in organic osmolyte is greatest for taurine (85%). (Adapted from Pasantes-Morales et al., 2002b)

absence of adequate concentrations of organic osmolytes may lead to central pontine myelinolysis and seizures (Kleinschmidt-DeMasters and Norenberg, 1981; Lien et al., 1991; Videen et al., 1995; Gross et al., 2001).

It is now well recognized that excess hydration can also lead to hyponatremia, for instance during military operations and marathon runs (O' Toole et al., 1995; Kratz et al., 2005). In 2002, 13% of Boston marathon runners were reported to have hyponatremia (Almond et al., 2005). Signs and symptoms for this include nausea, emesis and headaches which could be followed by seizures, respiratory arrest and coma (Farrell et al., 2003). Risk factors for hypo-osmotic neural swelling may also include pregnancy, elderly age and drugs such as selective serotonin reuptake inhibitors (e.g. paroxetine), antiepileptics and even the recreational drug, Ecstasy (Holmes et al., 1999; Jacob and Spinler, 2006). The blood-brain barrier imposes unique features of solvent and water movement into and out of the CNS. In cases where the blood-brain barrier is compromised and in 60% of meningiomas, treatment is complicated by brain edema (Bitzer et al., 2000).

The treatment of hyponatremia has changed little in the past decade. Intravenous injection of hypertonic solutions (3% saline or mannitol) is most frequently used to reduce increased intracranial pressure and cellular swelling (Wise and Chater, 1962; Munger, 2007). Conventional management techniques also include water restriction, demeclocycline, lithium and urea which have demonstrated variable efficacy and toxicity (Verbalis, 1993; Verhoeven et al., 2005; Kazama et al., 2007). Drugs such as furosemide (loop diuretics) are also

used in combination with hypertonic solutions, however, these treatments are not well-tolerated in all patients (Goldsmith, 2005). Therefore osmotic stress response must be considered within the context of intracellular water homeostasis which is influenced not only by extracellular environment but also by cellular metabolic activity. Arginine vasopressin (AVP) receptor antagonists (conivaptan, lixivaptan, and tolvaptan) are promising new therapeutic options whose safety and efficacy have been clinically established recently for some forms of hyponatremia (plasma  $\text{Na}^+$  decrease by 20 mM within 12 hrs-acute hyponatremia) (Cawley, 2007; Miyazaki et al., 2007).

On a molecular level, an understanding of the basic physiological processes and mechanisms of cell volume regulation is of crucial importance in neural cells. Osmolarity changes can have profound effects on cell-cell signaling because of the spatial relationship between neurons and astrocytes and as extracellular space becomes compromised due to swelling. Swelling can have dramatic consequences on diffusion of neurotransmitters such as glutamate, synaptic excitability, pH and amino acid homeostasis (Parkerson and Sontheimer, 2003). In such cases, non-perturbing organic osmolytes such as taurine serve as osmo-mediators between neural cells to critically regulate the composition of the extracellular space (Kimmelberg et al., 2000). *In vivo* experiments in hyponatremic rats and humans show a substantial decrease in organic osmolyte content in neural tissue, including a profound depletion of taurine with comparatively modest decreases in inorganic osmolytes (Lien et al., 1991; Massieu et al., 2004). Although astrocytes are considered to swell more

than neurons (Ordaz et al., 2004a), the release of osmolytes through VSOAC provides a means of neural communication between astrocytes and neurons even under physiological reductions in osmolarity (Mulligan and MacVicar, 2006).

## **Cellular and Molecular Mechanisms of Cell Swelling**

### ***Volume sensing***

RVD is a complex chain of events requiring a sensor to detect transient changes in cell volume, a signaling cascade to transduce information regarding volume changes into activation of pathways for osmolytes extrusion, and a “memory” of original cell volume to set the timing for inactivation (Pasantés-Morales et al., 2002a; Ordaz et al., 2004a). The nature of the initial critical step of volume sensing remains elusive. Among the possible mechanisms considered in the activation of the ion channel(s) are as follows: (1) direct activation by membrane stretch (Okada, 1997), (2) integrins and second messenger signals such as tyrosine kinases (Lambert and Hoffmann, 1994; Mongin and Orlov, 2001; Jakab et al., 2002), (3) changes in macromolecular crowding or cellular ionic strength (Voets et al., 1999; Burg, 2000) and (4) regulation through interaction of cytoskeleton (Papakonstanti et al., 2000).

However no one signaling mechanism can account for the volume sensitivity in all cells types. Mammalian cells have large membrane areas with numerous invaginations or ruffles that can accommodate for the increase in volume with no concomitant stretching of the membrane. Work on osmosensing in bacteria, their signal paths and membrane-based sensors have led to the



discovery of a group of proteins involved in osmoregulation termed the Osm proteins. These proteins share significant homology with *Drosophila* transient receptor potential (TRP) channels which are cation channels that mediate  $\text{Ca}^{2+}$  influx into cells and appear to play important roles in osmosensation and mechanosensation. Two mammalian TRP channels have recently been cloned and shown to be osmotically-regulated (Lietdke, 2005). Integrins are adhesion receptors that link the extracellular matrix to the actin cytoskeleton. It has been speculated that a change in cell volume may take place via modulation of integrin clustering that triggers intracellular signals (Pedersen et al., 2001). According to the macromolecular theory, the kinetics and equilibria of enzymes can be greatly influenced by small changes in the concentration of intracellular macromolecules and solutes. A 10% change in the concentration of intracellular proteins can lead to changes of up to a factor of ten in the thermodynamic activity of molecular regulatory mechanisms (Burg, 2000). However the evidence for these mechanisms along with the role of cytoskeleton in swelling is not entirely clear and may depend on the specific cell type (Moran et al., 1996b; Papakonstanti et al., 2000).

Thus, even though the initial volume-sensitive step awaits identification, it appears that unfolding of the plasma membrane, microdomains and reorganization of the cytoskeleton are potential elements of downstream signaling required by the cells to compensate for osmotic swelling.

### ***Signal transduction for swelling-induced VSOAC activity***

Depending on the specific cell type, swelling of the cell triggers many signal transduction mechanisms linked to the opening of the VSOAC (Okada, 1997; Calpham, 1998). These mechanisms are discussed below.

### ***Tyrosine Kinases***

Hypo-osmotic swelling elicits rapid and transient phosphorylation of a number of tyrosine kinases in a variety of cells such as cardiomyocytes, hepatoma, endothelial, epithelial cells and lymphocytes (Schliess et al., 1995; Sadoshima et al., 1996; Pasantes-Morales and Franco, 2002). The kinases Src, p125<sup>FAK</sup>, p38, JNK, p56<sup>LCK</sup> and mitogen activated protein kinases (MAPK) are among the phosphorylated proteins considered to be involved, depending on the cell type (Tilly et al., 1993; Crepel et al., 1998; Lepple-Wienhues et al., 1998). This has been addressed by testing the effects of various inhibitors of phosphotyrosine kinases (PTK). Tyrphostins, genistein and herbimycin strongly attenuate swelling activated Cl<sup>-</sup> and taurine efflux, although they exhibit different potencies according to the cell type (Nilius et al., 1997). Conversely, orthovanadate, a tyrosine phosphatase inhibitor, potentiates this pathway (Lang et al., 1998; Pasantes-Morales et al., 2000a). However a role for protein tyrosine phosphorylation in eliciting RVD is not universal and there is contradictory evidence in neural cells (Estevez et al., 1999, 2001; Franco et al., 2001; Heacock et al., 2004). Tyrosine kinases may also be involved in steps activated by processes occurring in association with swelling and volume regulation such as

cell adhesion, retraction and organization of the cytoskeleton, which makes it difficult to identify the precise step.

Nevertheless, a vast majority of studies suggest the role of tyrosine kinases in modulating the basal (swelling-activated) osmolyte release (Mongin et al., 1999; Delueze et al., 2000). Although the current literature provides evidence of one or more tyrosine phosphorylation steps for osmolyte efflux, the identity of specific kinase(s) and the precise step of the reaction are still unknown.

### ***Calcium, calmodulin and protein kinase C***

Exposure to hypotonic solutions can cause a rise in intracellular calcium in a variety of cells, whereas it remains constant in others (Lang et al., 1998). Swelling may increase calcium by both activation of calcium-permeable channels and calcium release from intracellular stores. Calcium ( $\text{Ca}^{2+}$ ) release from stores is presumably triggered by inositol phosphates or  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. Although a minimal amount of cell  $\text{Ca}^{2+} > 50 \text{ nM}$ , referred to as permissive  $\text{Ca}^{2+}$ , appears necessary for the activation of osmosensitive  $\text{Cl}^-$ /taurine fluxes, RVD and osmolytes effluxes are either  $\text{Ca}^{2+}$ -dependent or  $\text{Ca}^{2+}$ -independent depending on the cell type examined (Grinstein et al., 1982; O'Connor and Kimelberg, 1993; Szucs et al., 1996). Although  $\text{Ca}^{2+}$  is required for volume regulation in astrocytes (Mongin et al., 1999a), in neuroblastoma cells this requirement is only minimal for swelling-induced basal  $\text{Cl}^-$  and osmolyte release (Basavappa et al., 1995; Heacock et al., 2006).

Calmodulin inhibitors such as trifluoperazine and pimozide have been shown to block RVD and basal taurine release from astrocytes, Ehrlich and Hela cells (Li et al., 2002; Falktoft and Lambert, 2004) but they even inhibit efflux in cells where the calcium is below the threshold level for calmodulin activation (Szucs et al., 1996; Jorgensen et al., 1997). This suggests caution is warranted on conclusions reached based on these blockers as some of these inhibitors could directly affect the VSOAC. The increase in intracellular calcium may also activate  $\text{Ca}^{2+}$ -dependent kinases such as  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases and protein kinase C (PKC). PKC involvement in activating taurine efflux and RVD under swelling-activated basal conditions is also controversial and appears to be dependent on the cell type. It has been reported that chelerythrine inhibits the hypoosmotic amino acid release from cardiac cells, renal tubular cells and intestinal epithelial cells but not from rat cerebral cortex and glial cells. Conventional PKC isoforms are good candidates, however inhibitors presumably selective for cPKC isoforms show inhibition to a lesser extent than chelerythrine (Song et al., 1998; Estevez et al., 1999)

### ***Other signaling mechanisms***

In addition to the above signaling mechanisms, several other messengers have been implicated in activation of RVD. These include, phosphatidylinositol-3'-kinase, cytoskeleton remodeling and Rho effectors, formation of arachidonic acid and/or its metabolites (Tilly et al., 1996; Nilius et al., 1999, 2000; Lambert, 2004; Barfod et al., 2005)

These studies show interconnectivity of the vast signal transduction network involved in RVD. Phosphatidylinositol-3'-kinase has links to tyrosine-kinase membrane receptor, integrin-Focal Adhesion Kinase (FAK) pathway and also to small molecular weight (M.W) GTPases of the Rho family associated with remodeling of the cytoskeleton (Nilius et al., 1997). Cytochalasins that interfere with actin assembly show different results depending on the cell type (Shen et al., 1999); however it has been speculated that the cytoskeleton may play a role in insertion of cell volume regulatory channels into the plasma membrane (Lang et al., 1998). Rho kinase and myosin light chain kinases (MLCK) have also been reported to be involved in the activation of Cl<sup>-</sup> in endothelial cells, (Nilius et al., 1999) however Rho kinase inhibition does not affect taurine efflux in NIH3T3 cells (Pedersen et al., 2002).

Together these data suggest that the molecular mechanisms required to regulate cell volume may be extensive, complex and specific for every cell type. It may possibly involve the cytoskeleton and signal transduction cascade molecules including kinases, calcium and phospholipases. The experimental evidence provided in this thesis confirms the modulatory but not essential role of calcium and PKC in cell volume regulatory processes.

### **Regulation of Osmolyte Release: Role for Receptor Activation**

The mechanisms through which cell volume and osmolyte efflux can be modulated have recently received much attention. It has been observed that large, non-physiological decreases in extracellular osmolarity (>30%) are often

necessary to initiate intrinsically-induced osmolyte efflux *in vitro*. In contrast, *in vivo* volume changes can be observed during normal physiological changes in osmolarity and normal patterns of neuronal activity (Mulligan and MacVicar, 2007).

Previous reports suggest that osmolyte release can be enhanced by  $\text{Ca}^{2+}$  ionophores, phorbol esters (activators of PKC), or agents known to elevate cyclic AMP concentrations (Strange et al., 1996; Lambert, 2004). Intracellular application of non-hydrolyzable GTP analog, GTP $\gamma$ S has also been shown to activate swelling-induced  $\text{Cl}^-$  current which can be impaired by the addition of intracellular GDP $\beta$ S (Doroshenko, 1991; Voets et al., 1998). Although these results do not give any clue as to the type of G protein(s) that is involved, they raise the possibility that, *in vivo*, the activity of VSOAC may be under neurohumoral control.

Recently there have been a number of studies suggesting the role of G protein-coupled receptors (GPCRs) in the regulation of VSOAC. In non-neural cells, a few distinct receptors have previously been recognized to regulate swelling-activated chloride currents and organic osmolyte efflux. These include receptors for endothelin-2 in atrial cells (Du and Sorota, 2000), Epidermal Growth Factor in murine mammary cells (Abdullaev et al., 2003), thrombin and bradykinin in Ehrlich ascites tumor cells (Jorgensen et al., 1999), vasopressin in renal cells (Nakanishi et al., 1994), aldosterone in endothelial cells (Schneider et al., 1997),  $\text{H}_1$  histamine and Lyso-phosphatidic acid (LPA) receptors in HeLa cells (Lambert and Falktoft, 2000, 2004).

In contrast, in neural cells, vasopressin, an agonist at the V1 receptor, has been shown to play an important role in regulating RVD in pituicytes and astrocytes (Sarfraz and Fraser, 1999; Hertz et al., 2000; Rosso et al., 2004). In addition, two pharmacologically distinct receptors that had been extensively characterized in the regulation of volume-sensitive efflux of osmolytes at the initiation of this thesis include: M<sub>3</sub> muscarinic cholinergic (mAChR) in human SH-SY5Y neuroblastoma cells (Loveday et al., 2003; Heacock et al., 2004) and P2Y purinergic receptor in rat primary astrocytes (Mongin and Kimelberg, 2002). In each case, receptor activation reduced the “set-point” for osmolyte efflux to more physiologically relevant reductions in osmolarity (0-15%). Since these receptors can potentially couple to phospholipase C (PLC) activation and Ca<sup>2+</sup> mobilization, the possibility existed that there is a mechanistic link between these and other GPCRs and regulation of osmolytes efflux. Of particular importance is whether GPCR activation can modulate the release of organic osmolytes from neural cells and thereby contribute to RVD in response to hypo-osmotic stress.

### **G protein-coupled receptors**

The majority of hormones and neurotransmitters exert their biological effects through GPCRs. All GPCRs share one common structural element: a single polypeptide chain that forms seven transmembrane helices, connected by three intracellular and three extracellular loops. The transmembrane helical bundle forms a pocket where ligands can bind and interact with the receptor. Depending on the structure of the ligand, its binding can trigger (agonist) a

conformational change in the receptor molecule which affects its interaction with an associated, membrane-anchored G protein. G proteins act as molecular switches that transduce signals from more than 1,000 different receptors to intracellular effectors. A variety of effectors of G alpha subunits define different classes of G proteins, including adenylyl cyclase stimulating G proteins (Gs), adenylyl cyclase inhibiting G proteins (Gi), and phosphoinositide hydrolysis stimulating G proteins (Gq) (Birnbaumer et al., 1990). Gβγ also have a multitude of effectors.

Considering the physiological significance of regulating cell swelling in the brain and the fact that the CNS possesses a large number of GPCRs, the receptors yet identified to play an important role in osmoregulation in the CNS are as follows.

### ***Purinergic receptors***

There are eight human P2Y receptors currently recognized. These can couple to G proteins that are linked to IP<sub>3</sub> production and Ca<sup>2+</sup> mobilization, phosphatidylinositol 3-kinase (PI3K) via Gi, RhoA and Rho kinases, cAMP production and also are commonly associated with MAPK phosphorylation (Abbracchio et al., 2006). Multiple P2Y receptor subtypes are expressed in astrocytes, oligodendrocytes, and microglia and have been claimed to activate neuroprotective and pathophysiological mechanisms (Chorna et al., 2004; Abbracchio and Verderio, 2006). For cell volume regulation in neural cells, the purinergic receptor agonist, ATP, can enhance aspartate (used as a marker for



glutamate) efflux in primary cultures of astrocytes (Mongin and Kimelberg, 2002, 2005). ATP, preferentially via the P2Y1 and P2Y2 receptors that activate phospholipase C (PLC) -mediated  $Ca^{2+}$  mobilization, acts synergistically with cell swelling in enhancing aspartate release. ATP-induced organic osmolyte release was inhibited by cell shrinkage and anion channel blockers and was completely insensitive to 24-h pretreatment with tetanus toxin, in contrast to the astrocytic vesicular-like glutamate release (Mongin and Kimelberg, 2002). On the other hand, ATP itself is known to be released from cells during conditions that cause swelling (Junankar et al., 2002; Darby et al., 2003), which can in turn further modulate the release of other osmolytes such as taurine from neuronal populations (Li and Olson, 2004). This loss of organic osmolytes may lead to volume regulation in the CNS and is suggested to be the primary cellular mechanism for net transfer of taurine from neurons to astroglia observed in the osmotically swollen rat cerebellum *in situ* (Nagelhus et al., 1993). Besides the importance of this in neural cell communication, it must be kept in mind that high concentrations of the osmolyte, glutamate, can also cause excitotoxicity and pathological states.

### ***Muscarinic cholinergic receptors***

Molecular cloning studies have revealed the existence of five muscarinic subtypes. The even numbered receptors (M2 and M4) couple to inhibition of adenylyl cyclase through  $G_i/o$  and are pertussis toxin-sensitive. The odd numbered subtypes (M1, M3 and M5) couple through pertussis toxin-insensitive  $G_q/11$  and

mediate increases in inositol lipid turnover (Felder, 1995). The central nervous system contains a high density of muscarinic cholinergic receptors (mAChRs) that have been linked to a number of neuropsychiatric disorders such as Alzheimer's dementia, aging and Parkinson's disease (Dubois et al., 1983; Flynn et al., 1995). The presence of muscarinic cholinergic (M3) receptors has been documented in human 1321N1 astrocytomas and SH-SY5Y neuroblastoma cells and stimulation of these receptors is known to lead to activation of PLC and subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to form diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>) (Fisher and Heacock, 1988; Tang et al., 2002). Thus, agonist occupancy of this receptor subtype elicits the formation of two intracellular second messengers which are linked to activation of PKC and regulation of Ca<sup>2+</sup> homeostasis. Given the role that changes in protein phosphorylation and Ca<sup>2+</sup> play in cell volume regulation, the ability of these receptors to activate such signaling pathways assumes major importance.

In our laboratory, the ability of muscarinic cholinergic receptors (mAChRs) to regulate the volume-sensitive efflux of two organic osmolytes, namely taurine and D-aspartate, from human SH-SY5Y neuroblastoma cells has been examined (Loveday et al., 2002; Heacock et al., 2004). Inclusion of oxotremorine-M (Oxo-M), a muscarinic cholinergic agonist, not only results in a marked enhancement in the release of both osmolytes over a wide range of osmolarities but also lowers the threshold osmolarity at which the efflux of taurine and D-aspartate can occur. The results indicate that the activation of M3 mAChRs on SH-SY5Y neuroblastoma facilitate the cell's ability to respond to hypotonic stress by

potentiating organic osmolyte efflux through the VSOAC, and this enhancement seems to be dependent on  $\text{Ca}^{2+}$ , tyrosine kinases, and PKC activity (Loveday et al., 2002; Heacock et al., 2004).

My thesis further investigates the involvement of mAChRs in regulating the inorganic osmolyte,  $\text{Cl}^-$  as well as the ability of cholesterol to further modulate the taurine efflux response (Chapter 3, 4).

### ***Thrombin and its Protease-Activated Receptors***

One of the receptors upon which my thesis focuses is the protease-activated receptor (PAR) activated by thrombin. Thrombin is a serine protease cleaved from the proenzyme, prothrombin, by Factor Xa. The biological activities of thrombin include an essential component of the coagulation cascade, cleavage of fibrinogen to fibrin, platelet activation and stimulation of cellular responses involved in inflammation (Coughlin, 2000). Although it is difficult to detect thrombin levels in the brain, pro-thrombin RNA is found in various regions of the brain and the plasma levels of pro-thrombin are reportedly 1-5  $\mu\text{M}$  (Arai et al., 2006). During embryonic development when the blood-brain barrier is not yet intact, thrombin can directly affect neuronal cell growth and differentiation (Griffin et al., 2001). However, thrombin can be produced in the brain either immediately after cerebral hemorrhage or after breakdown of the blood-brain-barrier following brain injury such as trauma or ischemia (Lee et al., 1996). As a result thrombin can enhance the synthesis and secretion of nerve growth factor in glial cells,

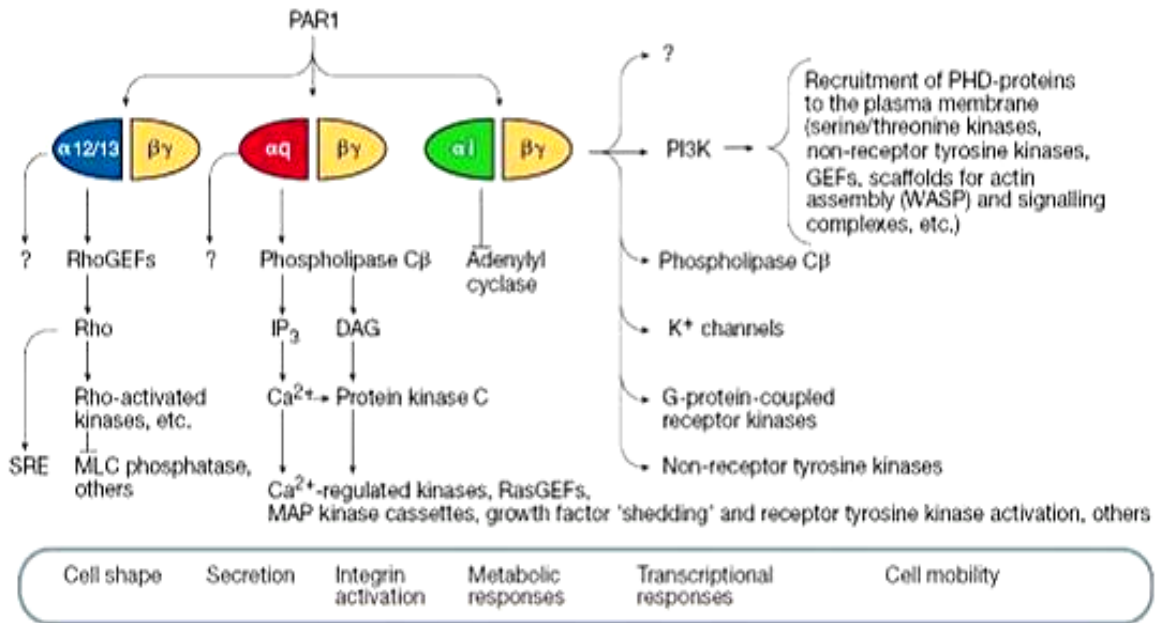
change the morphology of neurons and astrocytes and induce glial proliferation in the brain (Cavanaugh et al., 1990; Wang and Reiser, 2003).

Thrombin regulates cellular functions in a large variety of cells through a family of receptors known as Protease Activated Receptors (PARs). PARs belong to the GPCR superfamily and currently four members of the group (PAR1, PAR2, PAR3, and PAR4) have been identified. Thrombin activates PAR1, PAR3, and PAR4, whereas PAR2 is activated by trypsin. PARs are activated by an irreversible proteolytic cleavage of the receptor's extracellular N-terminus. This unmask a new N-terminus that functions as a "tethered peptide ligand," which then binds intra-molecularly to the receptor and initiates intracellular signal transduction (Macfarlane et al., 2001; Hollenberg, 2003). Synthetic peptides that mimic this tethered ligand comprise at least six amino acids and can activate the respective receptor, thereby by-passing the requirement for receptor proteolysis (Coughlin, 2000; Wang et al., 2002). Due to the unavailability of specific PAR antagonists, these peptides serve as important tools to probe the effects of different PAR receptor subtypes.

PARs are pleiotropic GPCRs capable of activating members of the  $G_{i/o}$ ,  $G_q$ , and  $G_{12/13}$  families of G proteins and hence a host of intracellular effectors depending on the cell type (Figure 1.5). PAR-1 can couple to inhibition of cAMP accumulation through  $G_i$  in a pertussis toxin-sensitive manner. Alternatively, it can couple to phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides through the  $G_q$  pathway. This leads to the production of inositol trisphosphate ( $IP_3$ ) which mobilizes intracellular  $Ca^{2+}$  and DAG, and in turn activates PKC

(Macfarlane et al., 2001; Hollenberg, 2003; Trejo, 2003). More recent studies have illustrated coupling of PAR1 to G<sub>12/13</sub>, modulating effectors such as Rho GEFs and small molecular weight (M.W) G-proteins such as Ras and Rho (Offermanns, S., et al, 1994; Majumdar et al., 1998). It can also activate other pathways such as the activation of Src tyrosine kinase, PI3K, MAPK, FAK and phospholipase A<sub>2</sub> (Choudhury et al., 1996; Wang, et al., al., 2002). Thrombin can promote a number of physiological responses due to its production of such various second messengers.

A role for thrombin in brain injury has only recently become apparent (Gingrich and Traynelis, 2000). Low concentrations of thrombin (50 pM to 100 nM) mediate neuroprotection against ischemia and environmental insults such as hypoglycemia, growth supplement deprivation, oxidative stress and  $\beta$ -amyloid toxicity (Vaughan, et al., 1995; Jiang et al., 2002; Guo et al., 2004) whereas prolonged administration of high concentrations of thrombin can cause neural degeneration and cell death (Striggow, et al., 2000; Hua et al., 2007). *In vivo*, pretreatment of the brain with a low dose of thrombin attenuates brain injury induced by cerebral hemorrhage or trauma (Xi et al., 1999, 2003; Masada et al., 2000). It has also been demonstrated that pro-thrombin mRNA and PAR1 and PAR3 receptors are up-regulated in various regions of the brain after environmental insults (Citron et al., 2000; Striggow, et al., 2001). Although these data indicate the regulatory importance of thrombin in the brain, the mechanism for this neuroprotection remains to be established. Therefore, thrombin is a multifunctional signaling molecule in the CNS and the characteristics of its effects



**Figure 1.5. Thrombin receptor signaling**

PAR-1 can couple to members of the G<sub>i</sub>, G<sub>q</sub> and G<sub>12/13</sub> families to impact a substantial network of signaling pathways as shown in the figure. The pleiotropic effects of PAR-1 activation are consistent with many of thrombin's diverse actions on cells. (Adapted from Coughlin, 2000)

in modulating osmolyte efflux is the major focus of this thesis. Although the role of thrombin in enhancing taurine and Cl<sup>-</sup> efflux was observed previously in myoblasts and endothelial cells (Sullivan et al., 1996; Manolopoulos et al., 1997a, b), the present thesis was the first to determine its role and mechanism in modulating osmolyte release in neuronal cells. These findings are of interest in terms of a potential regulatory action of these agents on the brain's adaptation to swelling.

### ***Lysophospholipid receptors***

During this thesis it was recognized by other members in our lab that lysophospholipids such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) can also facilitate the ability of SH-SY5Y neuroblastoma cells to respond to small reductions in osmolarity (Heacock et al., 2006). The principal effects of both LPA and S1P are mediated via a family of *edg* (endothelial differentiation gene) cell-surface receptors of which eight have been identified, three encoding receptors for LPA and five for S1P (Contos et al., 2000). SH-SY5Y cells express mRNA for *edg* 3, 4, 5, 7, and 8, which indicates the presence of LPA<sub>2</sub> and LPA<sub>3</sub> receptors (*edg* 4 and 7), and S1P, S1P<sub>2</sub>, and S1P<sub>3</sub> receptors (*edg* 8, 5, and 3, respectively) (Villullas et al., 2003). In the central nervous system, these lysophospholipids have also been implicated in neuronal growth, survival, differentiation, and cell signaling (for review, see Toman and Spiegel, 2002).

The addition of micromolar concentrations of LPA (10  $\mu$ M) or S1P (5  $\mu$ M) to SH-SY5Y cells elicits a robust increase in taurine efflux under isotonic as well as mild hypoosmotic conditions. The ability of LPA and S1P to increase osmolyte release was mimicked by certain analogs and other glycerophospholipids (Heacock et al., 2006) suggesting that several non-edg receptors that are phylogenetically closely related may also be involved (Kostenis, 2004). These results indicate that SH-SY5Y cells express multiple receptors that are activated by glycerophospholipids and sphingophospholipids that couple to the release of osmolytes. The involvement of a VSOAC in osmolyte release triggered by S1P or LPA is indicated by the ability of nonselective anion channel inhibitors, such as DDF and NPPB, as well as DCPIB, a highly selective inhibitor of VSOAC (Decher et al., 2001). Although a rise in  $[Ca^{2+}]_i$  is elicited by S1P or LPA, omission of extracellular  $Ca^{2+}$  does not significantly reduce the magnitude of taurine efflux in response to the addition of either lysophospholipid. However, a requirement for an intracellular pool of  $Ca^{2+}$  and PKC is essential for S1P- and LPA-stimulated osmolyte efflux. Pretreatment of SH-SY5Y cells with either pertussis toxin, cytochalasin D or toxin B, conditions previously established to disrupt the cytoskeleton in these cells (Linseman et al., 1998), does not alter basal or lysophospholipid-stimulated taurine efflux.

### **Role of Cholesterol in the Regulation of VSOAC**

Cholesterol is one of the major lipid components of the plasma membrane in mammalian cells making up to ~90% of the total cholesterol content in certain



cells (Lange et al., 1989). Changes in levels of cholesterol have a major impact on the physical properties of the membrane lipid bilayer such as ordering of phospholipids, changes in membrane fluidity, thickness, curvature and permeability (Xu and London, 2000). An increase in cholesterol, in turn, restricts the motion of phospholipids resulting in lipid ordering and a decrease in membrane fluidity (Cooper, 1978; Xu and London 2000). However, cholesterol distribution is heterogenous and the sterol is now known to be concentrated in cholesterol- and sphingomyelin-rich membrane domains (membrane rafts). The recent Keystone Symposium on lipid rafts and cell function (March 23-28, 2006 in Steamboat Springs, CO) defined membrane rafts or caveolae as “small (10 - 200 nm) heterogenous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” (Pike, 2006).

These lipid domains have recently received attention as they often serve as a molecular scaffold or regulatory platforms for signaling proteins such as PKC, G-proteins, Src tyrosine kinases or small GTPases Ras and Rho (Allen et al., 2007). Changes in membrane cholesterol content and association of the protein, caveolin, with lipid microdomains have also been demonstrated to modulate the function of a number of membrane transporters such as serotonin and ABC transporters (Scanlon et al., 2001; Dos-Santos et al., 2007), a number of distinct ion channels such as antibiotic ion channel gramicidin, large conductance  $\text{Ca}^{2+}$ -dependent K channel, L-type  $\text{Ca}^{2+}$  channel,  $\text{Kv}_{1.5}$  and TRP1 channels (Lundbaek et al., 1996; Lockwich et al., 2000; Martens et al., 2001) as

well as receptors such as oxytocin, 5-hydroxytryptamine 1A, neurokinin-1 and epidermal growth factor receptors (Burger et al., 2000).

The significance of cholesterol and lipid rafts in the regulation of VSOAC and taurine transport is controversial. It has been shown that swelling-induced Cl<sup>-</sup> conductance is limited in caveolin-1-deficient cells but that caveolin-1 protein expression facilitates the hypotonicity-induced Cl<sup>-</sup> current and taurine release in polarized colon cancer cells (Caco-2) and breast cancer cells (MCF-7 and T47D) (Trouet et al., 1999; Ullrich et al., 2006). In addition, transfection of mutant caveolin-1 in endothelial cells was found to reduce the hypotonicity-induced current (Trouet et al., 2001). In contrast, recent studies have demonstrated an inverse relation of VSOAC activity with a decrease in plasma membrane cholesterol content using methyl- $\beta$ -cyclodextrin (CD) (Levitan et al., 2000; Romanenko et al., 2004; Byfield et al., 2006).

CDs are cyclic oligosaccharides consisting of  $\alpha$ -(1-4)-linked D-glycopyranose units and exist as heptamers. These compounds, although water-soluble, have been long recognized as potent carriers for hydrophobic drugs, since they contain a hydrophobic cavity that can encapsulate various hydrophobic molecules. CDs are most widely used in cell biology to deplete sterols or serve as shuttles for sterol. They also function as agents known to perturb raft organization as well as decrease membrane fluidity by the extraction of cholesterol (Zidovetski and Levitan, 2007).

In bovine endothelial and intestinal epithelial cells CD-induced cholesterol depletion potentiated VSOAC current whereas an increase in cholesterol content

resulted in inhibition of swelling-activated  $\text{Cl}^-$  current (Levitan et al., 2000; Romanenko et al., 2004; Byfield et al., 2006; Lim et al., 2006). These data together suggest that cholesterol availability may provide another mechanism of cell volume regulation based on (a) the proximity of VSOAC to second messengers in lipid rafts (b) change in the biophysical properties of the membrane that could alter the pore properties, sorting or trafficking of the channels (Lim et al., 2006) or (c) change in the cellular distribution and content of F-actin (Klausen et al., 2006; Byfield et al., 2004).

A normal physiological level of cholesterol is essential for cell function and growth and is an important component of the white matter in the brain. Alterations in cholesterol content take place not only in conditions associated with disorders of cholesterol metabolism or transport such as Smith-Lemli-Opitz syndrome, Niemann-Pick type C, or Tangier disease (Maxfield and Tabas, 2005) but also by the intake of altered compositions of dietary lipids (Foot et al., 1982). Since the brain is the organ that is richest in cholesterol, the outcome of manipulating cholesterol and thereby intracellular signaling has been studied in various pathologies such as neuroinflammation, Huntington's disease, Alzheimer's disease and stroke (Zipp et al., 2007).

The activation of a plethora of events that may regulate VSOAC activity and organic osmolyte release after hypo-osmotic stress have to be analyzed systematically in an effort to dissect the interconnections and interdependence of different responses.

## Thesis Outline

For this thesis, human 1321N1 astrocytomas and SH-SY5Y neuroblastoma were used as model neural cell lines. 1321N1 and SH-SY5Y cell lines originated from highly malignant form of tumor in brain astrocytes and sympathetic neuroblasts of the peripheral nervous system, respectively. There are several advantages in using these cell lines, including their widespread use in previous research projects, ease of culture, but most importantly their homogeneity and presence of pharmacologically distinct receptors.

Recent studies from our laboratory showed that muscarinic receptors (M3) on SH-SY5Y cells regulate the efflux of organic osmolytes through the VSOAC. However, little was known about the regulation of VSOAC by other GPCRs in these neural cells and the mechanisms involved. My thesis was therefore focused on **understanding the GPCR mechanisms of regulating osmolyte efflux through the VSOAC in neural cells**. This dissertation work is comprised of three data chapters (Chapters 2, 3, 4).

After an extensive investigation of receptor ligands that enhance organic osmolyte efflux in response to hypoosmotic stress, I discovered that thrombin produced the most robust potentiation in organic osmolyte efflux. Hence, the focus of chapter 2 is the observation that sub-nanomolar concentrations of thrombin, mainly via the PAR-1 receptor, can robustly enhance the release of the organic osmolyte, taurine, through the VSOAC from 1321N1 astrocytoma cells. To the best of my knowledge, this was the first demonstration that thrombin in neural cells can enhance taurine efflux at physiologically relevant reductions in

osmolarity. I performed a detailed biochemical and pharmacological characterization of thrombin-stimulated taurine release and demonstrated that both intracellular calcium and protein kinase C (PKC) are required for thrombin-induced taurine release. This work has been published in the *Journal of Pharmacology and Experimental Therapeutics* 2005; 315 (2):755-63.

The goals of chapter 3 were two-fold: first, to determine whether the release of the inorganic osmolyte, chloride, from hypotonically stressed SH-SY5Y neuroblastoma cells was, like that of taurine, subject to receptor regulation, and second, to evaluate whether these two osmolytes are released from the cells via distinct or similar mechanisms. This was important in view of the recent controversy that organic osmolytes and Cl<sup>-</sup> may exit through separate channels instead of a common VSOAC. Radioactive <sup>125</sup>I<sup>-</sup> (used as a tracer for Cl<sup>-</sup>) and [<sup>3</sup>H]taurine was monitored for these studies. I found that although thrombin and Oxotremorine-M enhanced both taurine and <sup>125</sup>I<sup>-</sup> efflux, the kinetics and magnitude of <sup>125</sup>I<sup>-</sup> release were greater. Inclusion of anion channel blockers also attenuated the release of both <sup>125</sup>I<sup>-</sup> and taurine under basal- and receptor-stimulated conditions. Although PAR-1 and mAChR-stimulated taurine efflux was attenuated by either a depletion of intracellular Ca<sup>2+</sup> or inhibition of PKC, the enhanced release of <sup>125</sup>I<sup>-</sup> was less dependent on these parameters. Together, these results indicate that although the release of these two osmolytes from SH-SY5Y cells may occur via pharmacologically similar membrane channels, the receptor-mediated release of taurine and <sup>125</sup>I<sup>-</sup> is differentially regulated by PKC

and  $\text{Ca}^{2+}$  availability. This work has been published in the *Journal of Pharmacology and Experimental Therapeutics* 2007; 320(3):1068-77.

In chapter 4, I attempt to further elucidate the regulation of receptor-mediated taurine release through the VSOAC by testing the role of cholesterol in SH-SY5Y neuroblastoma cells. Cholesterol depletion, elicited by methyl- $\beta$ -cyclodextrin (CD), enhanced both the basal (swelling-induced) and receptor-stimulated release of taurine. The stimulatory effect of cholesterol-depletion was more pronounced for all the receptors tested (PAR-1, mAChR and S1P) than for the basal release. Therefore, the ability of cholesterol depletion to regulate agonist potency and efficacy was tested by means of studies with PAR-1 and mAChR receptor subtypes. To ensure that the CD-induced stimulatory effects were specific, experiments were performed after repleting the cells with cholesterol or substituting it with its chiral analog, epi-cholesterol. To further determine if the cholesterol depletion effects on the basal- and receptor-stimulated taurine release were due to disruption of lipid rafts, other agents such as cholesterol oxidase, sphingomyelinase and filipin, which also perturb raft organization, were tested. The results provide evidence that cholesterol serves to further regulate receptor-mediated taurine release most likely due to the selective effects of the sterol on membrane properties such as fluidity rather than through lipid rafts.

The data discussed in Chapters 2, 3, and 4 demonstrate the importance of receptor and cholesterol regulation of osmolyte release through the Volume Sensitive Organic osmolyte and Anion Channel. Also, the novel role of thrombin

in osmoregulation, adds to the growing list of functions attributed to this protease in CNS physiology and pathology.

## References

- Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA and Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 58:281-341.
- Abbracchio MP and Verderio C (2006) Pathophysiological roles of P2 receptors in glial cells. *Novartis Found Symp* 276:91-103; discussion 103-112, 275-181.
- Abdullaev IF, Rudkouskaya A, Schools GP, Kimelberg HK and Mongin AA (2006) Pharmacological comparison of swelling-activated excitatory amino acid release and Cl<sup>-</sup> currents in cultured rat astrocytes. *J Physiol* 572:677-689.
- Abdullaev IF, Sabirov RZ and Okada Y (2003) Upregulation of swelling-activated Cl<sup>-</sup> channel sensitivity to cell volume by activation of EGF receptors in murine mammary cells. *J Physiol* 549:749-758.
- Aerts L and Van Assche FA (2002) Taurine and taurine-deficiency in the perinatal period. *J Perinat Med* 30:281-286.
- Agre P and Kozono D (2003) Aquaporin water channels: molecular mechanisms for human diseases. *FEBS Lett* 555:72-78.
- Almond CS, Shin AY, Fortescue EB, Mannix RC, Wypij D, Binstadt BA, Duncan CN, Olson DP, Salerno AE, Newburger JW and Greenes DS (2005) Hyponatremia among runners in the Boston Marathon. *N Engl J Med* 352:1550-1556.
- Anderson RJ, Chung HM, Kluge R and Schrier RW (1985) Hyponatremia: a prospective analysis of its epidemiology and the pathogenetic role of vasopressin. *Ann Intern Med* 102:164-168.
- Arai T, Miklossy J, Klegeris A, Guo JP and McGeer PL (2006) Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. *J Neuropathol Exp Neurol* 65:19-25.
- Aruoma OI, Halliwell B, Hoey BM and Butler J (1988) The antioxidant action of taurine, hypotaurine and their metabolic precursors. *Biochem J* 256:251-255.
- Barfield JP, Yeung CH and Cooper TG (2005) Characterization of potassium channels involved in volume regulation of human spermatozoa. *Mol Hum Reprod* 11:891-897.



- Barfod ET, Moore AL, Melnick RF and Lidofsky SD (2005) Src regulates distinct pathways for cell volume control through Vav and phospholipase Cgamma. *J Biol Chem* 280:25548-25557.
- Basavappa S, Chartouni V, Kirk K, Prpic V, Ellory JC and Mangel AW (1995) Swelling-induced chloride currents in neuroblastoma cells are calcium dependent. *J Neurosci* 15:3662-3666.
- Berl T (1990) Treating hyponatremia: damned if we do and damned if we don't. *Kidney Int* 37:1006-1018.
- Best L, Yates AP, Decher N, Steinmeyer K and Nilius B (2004) Inhibition of glucose-induced electrical activity in rat pancreatic beta-cells by DCPIB, a selective inhibitor of volume-sensitive anion currents. *Eur J Pharmacol* 489:13-19.
- Bildin VN, Yang H, Crook RB, Fischbarg J and Reinach PS (2000) Adaptation by corneal epithelial cells to chronic hypertonic stress depends on upregulation of Na:K:2Cl cotransporter gene and protein expression and ion transport activity. *J Membr Biol* 177:41-50.
- Birdsall TC (1998) Therapeutic applications of taurine. *Altern Med Rev* 3:128-136.
- Birnbaumer L, Abramowitz J and Brown AM (1990) Receptor-effector coupling by G proteins. *Biochim Biophys Acta* 1031:163-224.
- Bitzer M, Nagele T, Geist-Barth B, Klose U, Gronewaller E, Morgalla M, Heiss E and Voigt K (2000) Role of hydrodynamic processes in the pathogenesis of peritumoral brain edema in meningiomas. *J Neurosurg* 93:594-604.
- Burg MB (2000) Macromolecular crowding as a cell volume sensor. *Cell Physiol Biochem* 10:251-256.
- Burger K, Gimpl G and Fahrenholz F (2000) Regulation of receptor function by cholesterol. *Cell Mol Life Sci* 57:1577-1592.
- Bussmann C, Bast T and Rating D (2001) Hyponatraemia in children with acute CNS disease: SIADH or cerebral salt wasting? *Childs Nerv Syst* 17:58-62; discussion 63.
- Bustamante J, Lobo MV, Alonso FJ, Mukala NT, Gine E, Solis JM, Tamarit-Rodriguez J and Martin Del Rio R (2001) An osmotic-sensitive taurine pool is localized in rat pancreatic islet cells containing glucagon and somatostatin. *Am J Physiol Endocrinol Metab* 281:E1275-1285.

- Byfield FJ, Aranda-Espinoza H, Romanenko VG, Rothblat GH and Levitan I (2004) Cholesterol depletion increases membrane stiffness of aortic endothelial cells. *Biophys J* 87:3336-3343.
- Byfield FJ, Hoffman BD, Romanenko VG, Fang Y, Crocker JC and Levitan I (2006) Evidence for the role of cell stiffness in modulation of volume-regulated anion channels. *Acta Physiol (Oxf)* 187:285-294.
- Cavanaugh KP, Gurwitz D, Cunningham DD and Bradshaw RA (1990) Reciprocal modulation of astrocyte stellation by thrombin and protease nexin-1. *J Neurochem* 54:1735-1743.
- Cawley MJ (2007) Hyponatremia: current treatment strategies and the role of vasopressin antagonists. *Ann Pharmacother* 41:840-850.
- Chorna NE, Santiago-Perez LI, Erb L, Seye CI, Neary JT, Sun GY, Weisman GA and Gonzalez FA (2004) P2Y receptors activate neuroprotective mechanisms in astrocytic cells. *J Neurochem* 91:119-132.
- Choudhury GG, Marra F and Abboud HE (1996) Thrombin stimulates association of src homology domain containing adaptor protein Nck with pp125FAK. *Am J Physiol* 270:F295-300.
- Citron BA, Smirnova IV, Arnold PM and Festoff BW (2000) Upregulation of neurotoxic serine proteases, prothrombin, and protease-activated receptor 1 early after spinal cord injury. *J Neurotrauma* 17:1191-1203.
- Clapham DE (1998) The list of potential volume-sensitive chloride currents continues to swell (and shrink). *J Gen Physiol* 111:623-624.
- Contos JJ, Ishii I and Chun J (2000) Lysophosphatidic acid receptors. *Mol Pharmacol* 58:1188-1196.
- Cooper RA (1978) Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells. *J Supramol Struct* 8:413-430.
- Coughlin SR (2000) Thrombin signalling and protease-activated receptors. *Nature* 407:258-264.
- Crepel V, Panenka W, Kelly ME and MacVicar BA (1998) Mitogen-activated protein and tyrosine kinases in the activation of astrocyte volume-activated chloride current. *J Neurosci* 18:1196-1206.
- Cserr HF, DePasquale M, Nicholson C, Patlak CS, Pettigrew KD and Rice ME (1991) Extracellular volume decreases while cell volume is maintained by ion uptake in rat brain during acute hypernatremia. *J Physiol* 442:277-295.

- Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Rev* 53:121-147.
- Darby M, Kuzmiski JB, Panenka W, Feighan D and MacVicar BA (2003) ATP released from astrocytes during swelling activates chloride channels. *J Neurophysiol* 89:1870-1877.
- Decher N, Lang HJ, Nilius B, Bruggemann A, Busch AE and Steinmeyer K (2001) DCPIB is a novel selective blocker of I(Cl,swell) and prevents swelling-induced shortening of guinea-pig atrial action potential duration. *Br J Pharmacol* 134:1467-1479.
- Deleuze C, Duvoid A, Moos FC and Hussy N (2000) Tyrosine phosphorylation modulates the osmosensitivity of volume-dependent taurine efflux from glial cells in the rat supraoptic nucleus. *J Physiol* 523 Pt 2:291-299.
- Deutsch C and Chen LQ (1993) Heterologous expression of specific K<sup>+</sup> channels in T lymphocytes: functional consequences for volume regulation. *Proc Natl Acad Sci U S A* 90:10036-10040.
- Doroshenko P (1991) Second messengers mediating activation of chloride current by intracellular GTP gamma S in bovine chromaffin cells. *J Physiol* 436:725-738.
- Dos Santos SM, Weber CC, Franke C, Muller WE and Eckert GP (2007) Cholesterol: Coupling between membrane microenvironment and ABC transporter activity. *Biochem Biophys Res Commun* 354:216-221.
- Du XY and Sorota S (2000) Cardiac swelling-induced chloride current is enhanced by endothelin. *J Cardiovasc Pharmacol* 35:769-776.
- Duan D, Winter C, Cowley S, Hume JR and Horowitz B (1997) Molecular identification of a volume-regulated chloride channel. *Nature* 390:417-421.
- Dubois B, Ruberg M, Javoy-Agid F, Ploska A and Agid Y (1983) A subcortico-cortical cholinergic system is affected in Parkinson's disease. *Brain Res* 288:213-218.
- Estevez AY, Bond T and Strange K (2001) Regulation of I(Cl,swell) in neuroblastoma cells by G protein signaling pathways. *Am J Physiol Cell Physiol* 281:C89-98.
- Estevez AY, O'Regan MH, Song D and Phillis JW (1999) Hyposmotically induced amino acid release from the rat cerebral cortex: role of phospholipases and protein kinases. *Brain Res* 844:1-9.

- Falktoft B and Lambert IH (2004) Ca<sup>2+</sup>-mediated potentiation of the swelling-induced taurine efflux from HeLa cells: on the role of calmodulin and novel protein kinase C isoforms. *J Membr Biol* 201:59-75.
- Farrell DJ and Bower L (2003) Fatal water intoxication. *J Clin Pathol* 56:803-804.
- Felder CC (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *Faseb J* 9:619-625.
- Fisher SK and Heacock AM (1988) A putative M3 muscarinic cholinergic receptor of high molecular weight couples to phosphoinositide hydrolysis in human SK-N-SH neuroblastoma cells. *J Neurochem* 50:984-987.
- Flynn DD, Ferrari-DiLeo G, Levey AI and Mash DC (1995) Differential alterations in muscarinic receptor subtypes in Alzheimer's disease: implications for cholinergic-based therapies. *Life Sci* 56:869-876.
- Foot M, Cruz TF and Clandinin MT (1982) Influence of dietary fat on the lipid composition of rat brain synaptosomal and microsomal membranes. *Biochem J* 208:631-640.
- Franciolini F and Nonner W (1987) Anion and cation permeability of a chloride channel in rat hippocampal neurons. *J Gen Physiol* 90:453-478.
- Franco R, Torres-Marquez ME and Pasantes-Morales H (2001) Evidence for two mechanisms of amino acid osmolyte release from hippocampal slices. *Pflugers Arch* 442:791-800.
- Gaull GE (1989) Taurine in pediatric nutrition: review and update. *Pediatrics* 83:433-442.
- Gingrich MB and Traynelis SF (2000) Serine proteases and brain damage - is there a link? *Trends Neurosci* 23:399-407.
- Goldsmith SR (2005) Current treatments and novel pharmacologic treatments for hyponatremia in congestive heart failure. *Am J Cardiol* 95:14B-23B.
- Griffin CT, Srinivasan Y, Zheng YW, Huang W and Coughlin SR (2001) A role for thrombin receptor signaling in endothelial cells during embryonic development. *Science* 293:1666-1670.
- Grinstein S, Dupre A and Rothstein A (1982) Volume regulation by human lymphocytes. Role of calcium. *J Gen Physiol* 79:849-868.
- Gross P (2001) Treatment of severe hyponatremia. *Kidney Int* 60:2417-2427.

- Guizouarn H, Motais R, Garcia-Romeu F and Borgese F (2000) Cell volume regulation: the role of taurine loss in maintaining membrane potential and cell pH. *J Physiol* 523 Pt 1:147-154.
- Gullans SR and Verbalis JG (1993) Control of brain volume during hyperosmolar and hypoosmolar conditions. *Annu Rev Med* 44:289-301.
- Guo H, Liu D, Gelbard H, Cheng T, Insalaco R, Fernandez JA, Griffin JH and Zlokovic BV (2004) Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. *Neuron* 41:563-572.
- Gupta RC, Win T and Bittner S (2005) Taurine analogues; a new class of therapeutics: retrospect and prospects. *Curr Med Chem* 12:2021-2039.
- Hall JE, Guyton AC and Brands MW (1996) Pressure-volume regulation in hypertension. *Kidney Int Suppl* 55:S35-41.
- Han X, Patters AB, Jones DP, Zelikovic I and Chesney RW (2006) The taurine transporter: mechanisms of regulation. *Acta Physiol (Oxf)* 187:61-73.
- Hardy SP, Goodfellow HR, Valverde MA, Gill DR, Sepulveda V and Higgins CF (1995) Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume-activated chloride channels. *Embo J* 14:68-75.
- Haussinger D and Gerok W (1994) Role of the cellular hydration state for cellular function: physiological and pathophysiological aspects. *Adv Exp Med Biol* 368:33-44.
- Haussinger D, Lang F and Gerok W (1994a) Regulation of cell function by the cellular hydration state. *Am J Physiol* 267:E343-355.
- Haussinger D, Laubenberger J, vom Dahl S, Ernst T, Bayer S, Langer M, Gerok W and Hennig J (1994b) Proton magnetic resonance spectroscopy studies on human brain myo-inositol in hypo-osmolarity and hepatic encephalopathy. *Gastroenterology* 107:1475-1480.
- Hayashi T, Ishida Y, Miyashita T, Kiyokawa H, Kimura A and Kondo T (2005) Fatal water intoxication in a schizophrenic patient--an autopsy case. *J Clin Forensic Med* 12:157-159.
- Heacock AM, Dodd MS and Fisher SK (2006) Regulation of volume-sensitive osmolyte efflux from human SH-SY5Y neuroblastoma cells following activation of lysophospholipid receptors. *J Pharmacol Exp Ther* 317:685-693.
- Heacock AM, Kerley D, Gurda GT, VanTroostenberghe AT and Fisher SK (2004) Potentiation of the osmosensitive release of taurine and D-aspartate from

- SH-SY5Y neuroblastoma cells after activation of M3 muscarinic cholinergic receptors. *J Pharmacol Exp Ther* 311:1097-1104.
- Hertz L, Chen Y and Spatz M (2000) Effects of arginine vasopressin on water space in astrocytes and in whole brain. *Am J Physiol Endocrinol Metab* 278:E1175-1176.
- Hoffmann EK and Lambert IH (1983) Amino acid transport and cell volume regulation in Ehrlich ascites tumour cells. *J Physiol* 338:613-625.
- Hollenberg MD (2003) Proteinase-mediated signaling: proteinase-activated receptors (PARs) and much more. *Life Sci* 74:237-246.
- Hollenberg MD and Compton SJ (2002) International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol Rev* 54:203-217.
- Holmes SB, Banerjee AK and Alexander WD (1999) Hyponatraemia and seizures after ecstasy use. *Postgrad Med J* 75:32-33.
- Hoorn EJ, Lindemans J and Zietse R (2006) Development of severe hyponatraemia in hospitalized patients: treatment-related risk factors and inadequate management. *Nephrol Dial Transplant* 21:70-76.
- Hua Y, Keep RF, Hoff JT and Xi G (2007) Brain injury after intracerebral hemorrhage: the role of thrombin and iron. *Stroke* 38:759-762.
- Hussy N, Deleuze C, Bres V and Moos FC (2000) New role of taurine as an osmomediator between glial cells and neurons in the rat supraoptic nucleus. *Adv Exp Med Biol* 483:227-237.
- Hussy N, Deleuze C, Pantaloni A, Desarmenien MG and Moos F (1997) Agonist action of taurine on glycine receptors in rat supraoptic magnocellular neurones: possible role in osmoregulation. *J Physiol* 502 (Pt 3):609-621.
- Huxtable RJ (1989) Taurine in the central nervous system and the mammalian actions of taurine. *Prog Neurobiol* 32:471-533.
- Huxtable RJ (2000) Expanding the circle 1975-1999: sulfur biochemistry and insights on the biological functions of taurine. *Adv Exp Med Biol* 483:1-25.
- Inoue H, Mori S, Morishima S and Okada Y (2005) Volume-sensitive chloride channels in mouse cortical neurons: characterization and role in volume regulation. *Eur J Neurosci* 21:1648-1658.
- Jackson PS, Morrison R and Strange K (1994) The volume-sensitive organic osmolyte-anion channel VSOAC is regulated by nonhydrolytic ATP binding. *Am J Physiol* 267:C1203-1209.

- Jackson PS and Strange K (1993) Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. *Am J Physiol* 265:C1489-1500.
- Jackson PS and Strange K (1995) Characterization of the voltage-dependent properties of a volume-sensitive anion conductance. *J Gen Physiol* 105:661-676.
- Jacob S and Spinler SA (2006) Hyponatremia associated with selective serotonin-reuptake inhibitors in older adults. *Ann Pharmacother* 40:1618-1622.
- Jacobsen JG and Smith LH (1968) Biochemistry and physiology of taurine and taurine derivatives. *Physiol Rev* 48:424-511.
- Jakab M, Furst J, Gschwentner M, Botta G, Garavaglia ML, Bazzini C, Rodighiero S, Meyer G, Eichmueller S, Woll E, Chwatal S, Ritter M and Paulmichl M (2002) Mechanisms sensing and modulating signals arising from cell swelling. *Cell Physiol Biochem* 12:235-258.
- Jentsch TJ (2002) Chloride channels are different. *Nature* 415:276-277.
- Jentsch TJ, Stein V, Weinreich F and Zdebik AA (2002) Molecular structure and physiological function of chloride channels. *Physiol Rev* 82:503-568.
- Jiang Y, Wu J, Hua Y, Keep RF, Xiang J, Hoff JT and Xi G (2002) Thrombin-receptor activation and thrombin-induced brain tolerance. *J Cereb Blood Flow Metab* 22:404-410.
- Jin NG, Kim JK, Yang DK, Cho SJ, Kim JM, Koh EJ, Jung HC, So I and Kim KW (2003) Fundamental role of ClC-3 in volume-sensitive Cl<sup>-</sup> channel function and cell volume regulation in AGS cells. *Am J Physiol Gastrointest Liver Physiol* 285:G938-948.
- Johansson I, Karlsson M, Johanson U, Larsson C and Kjellbom P (2000) The role of aquaporins in cellular and whole plant water balance. *Biochim Biophys Acta* 1465:324-342.
- Jorgensen NK, Christensen S, Harbak H, Brown AM, Lambert IH, Hoffmann EK and Simonsen LO (1997) On the role of calcium in the regulatory volume decrease (RVD) response in Ehrlich mouse ascites tumor cells. *J Membr Biol* 157:281-299.
- Jorgensen NK, Petersen SF and Hoffmann EK (1999) Thrombin-, bradykinin-, and arachidonic acid-induced Ca<sup>2+</sup> signaling in Ehrlich ascites tumor cells. *Am J Physiol* 276:C26-37.

- Junankar PR, Karjalainen A and Kirk K (2002) The role of P2Y1 purinergic receptors and cytosolic Ca<sup>2+</sup> in hypotonically activated osmolyte efflux from a rat hepatoma cell line. *J Biol Chem* 277:40324-40334.
- Junankar PR and Kirk K (2000) Organic osmolyte channels: a comparative view. *Cell Physiol Biochem* 10:355-360.
- Kaya K and Sano T (1991) Definition of total biosynthesis pathway of taurolipids in Tetrahymena cells. *Biochim Biophys Acta* 1084:101-104.
- Kazama I, Arata T, Michimata M, Hatano R, Suzuki M, Miyama N, Sanada S, Sato A, Satomi S, Ejima Y, Sasaki S and Matsubara M (2007) Lithium effectively complements vasopressin V2 receptor antagonist in the treatment of hyponatraemia of SIADH rats. *Nephrol Dial Transplant* 22:68-76.
- Kettenmann H (1987) K<sup>+</sup> and Cl<sup>-</sup> uptake by cultured oligodendrocytes. *Can J Physiol Pharmacol* 65:1033-1037.
- Kimelberg HK (1995) Current concepts of brain edema. Review of laboratory investigations. *J Neurosurg* 83:1051-1059.
- Kimelberg HK, Cai Z, Schools G and Zhou M (2000) Acutely isolated astrocytes as models to probe astrocyte functions. *Neurochem Int* 36:359-367.
- King LS, Kozono D and Agre P (2004) From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 5:687-698.
- Kirk K and Strange K (1998) Functional properties and physiological roles of organic solute channels. *Annu Rev Physiol* 60:719-739.
- Klausen TK, Hougaard C, Hoffmann EK and Pedersen SF (2006) Cholesterol modulates the volume-regulated anion current in Ehrlich-Lette ascites cells via effects on Rho and F-actin. *Am J Physiol Cell Physiol* 291:C757-771.
- Kleinschmidt-DeMasters BK and Norenberg MD (1981) Rapid correction of hyponatremia causes demyelination: relation to central pontine myelinolysis. *Science* 211:1068-1070.
- Kostenis E (2004) Novel clusters of receptors for sphingosine-1-phosphate, sphingosylphosphorylcholine, and (lyso)-phosphatidic acid: new receptors for "old" ligands. *J Cell Biochem* 92:923-936.
- Krapivinsky GB, Ackerman MJ, Gordon EA, Krapivinsky LD and Clapham DE (1994) Molecular characterization of a swelling-induced chloride conductance regulatory protein, pICln. *Cell* 76:439-448.



- Kratz A, Siegel AJ, Verbalis JG, Adner MM, Shirey T, Lee-Lewandrowski E and Lewandrowski KB (2005) Sodium status of collapsed marathon runners. *Arch Pathol Lab Med* 129:227-230.
- Kreisman NR and Olson JE (2003) Taurine enhances volume regulation in hippocampal slices swollen osmotically. *Neuroscience* 120:635-642.
- Krogh A (1939) The Active Uptake of Ions into Cells and Organisms. *Proc Natl Acad Sci U S A* 25:275-277.
- Lambert IH (2004) Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. *Neurochem Res* 29:27-63.
- Lambert IH and Falktoft B (2000) Lysophosphatidylcholine induces taurine release from HeLa cells. *J Membr Biol* 176:175-185.
- Lambert IH and Hoffmann EK (1993) Regulation of taurine transport in Ehrlich ascites tumor cells. *J Membr Biol* 131:67-79.
- Lambert IH and Hoffmann EK (1994) Cell swelling activates separate taurine and chloride channels in Ehrlich mouse ascites tumor cells. *J Membr Biol* 142:289-298.
- Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E and Haussinger D (1998a) Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 78:247-306.
- Lang F, Busch GL and Volkl H (1998b) The diversity of volume regulatory mechanisms. *Cell Physiol Biochem* 8:1-45.
- Lang F, Lepple-Wienhues A, Paulmichl M, Szabo I, Siemen D and Gulbins E (1998c) Ion channels, cell volume, and apoptotic cell death. *Cell Physiol Biochem* 8:285-292.
- Lange Y, Ye J and Steck TL (1998) Circulation of cholesterol between lysosomes and the plasma membrane. *J Biol Chem* 273:18915-18922.
- Lee KR, Colon GP, Betz AL, Keep RF, Kim S and Hoff JT (1996) Edema from intracerebral hemorrhage: the role of thrombin. *J Neurosurg* 84:91-96.
- Lehmann A and Hansson E (1987) Amino acid content in astroglial primary cultures from different brain regions during cultivation. *Neurochem Res* 12:797-800.
- Lepple-Wienhues A, Szabo I, Laun T, Kaba NK, Gulbins E and Lang F (1998) The tyrosine kinase p56lck mediates activation of swelling-induced chloride channels in lymphocytes. *J Cell Biol* 141:281-286.

- Levitan I, Christian AE, Tulenko TN and Rothblat GH (2000) Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. *J Gen Physiol* 115:405-416.
- Li G, Liu Y and Olson JE (2002) Calcium/calmodulin-modulated chloride and taurine conductances in cultured rat astrocytes. *Brain Res* 925:1-8.
- Li G and Olson JE (2004) Extracellular ATP activates chloride and taurine conductances in cultured hippocampal neurons. *Neurochem Res* 29:239-246.
- Li X, Shimada K, Showalter LA and Weinman SA (2000) Biophysical properties of CIC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. *J Biol Chem* 275:35994-35998.
- Liedtke W (2005) TRPV4 as osmosensor: a transgenic approach. *Pflugers Arch* 451:176-180.
- Lien YH, Shapiro JI and Chan L (1991) Study of brain electrolytes and organic osmolytes during correction of chronic hyponatremia. Implications for the pathogenesis of central pontine myelinolysis. *J Clin Invest* 88:303-309.
- Lim CH, Schoonderwoerd K, Kleijer WJ, de Jonge HR and Tilly BC (2006) Regulation of the cell swelling-activated chloride conductance by cholesterol-rich membrane domains. *Acta Physiol (Oxf)* 187:295-303.
- Linseman DA, McEwen EL, Sorensen SD and Fisher SK (1998) Cytoskeletal and phosphoinositide requirements for muscarinic receptor signaling to focal adhesion kinase and paxillin. *J Neurochem* 70:940-950.
- Lockwich TP, Liu X, Singh BB, Jadlovec J, Weiland S and Ambudkar IS (2000) Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J Biol Chem* 275:11934-11942.
- Lombardini JB (1991) Taurine: retinal function. *Brain Res Brain Res Rev* 16:151-169.
- Loveday D, Heacock AM and Fisher SK (2003) Activation of muscarinic cholinergic receptors enhances the volume-sensitive efflux of myo-inositol from SH-SY5Y neuroblastoma cells. *J Neurochem* 87:476-486.
- Lundbaek JA, Birn P, Girshman J, Hansen AJ and Andersen OS (1996) Membrane stiffness and channel function. *Biochemistry* 35:3825-3830.
- Macfarlane SR, Seatter MJ, Kanke T, Hunter GD and Plevin R (2001) Proteinase-activated receptors. *Pharmacol Rev* 53:245-282.

- Majumdar M, Seasholtz TM, Goldstein D, de Lanerolle P and Brown JH (1998) Requirement for Rho-mediated myosin light chain phosphorylation in thrombin-stimulated cell rounding and its dissociation from mitogenesis. *J Biol Chem* 273:10099-10106.
- Manolopoulos GV, Prenen J, Droogmans G and Nilius B (1997a) Thrombin potentiates volume-activated chloride currents in pulmonary artery endothelial cells. *Pflugers Arch* 433:845-847.
- Manolopoulos VG, Droogmans G and Nilius B (1997b) Hypotonicity and thrombin activate taurine efflux in BC3H1 and C2C12 myoblasts that is down regulated during differentiation. *Biochem Biophys Res Commun* 232:74-79.
- Marnela KM, Morris HR, Panico M, Timonen M and Lahdesmaki P (1985) Glutamyl-aurine is the predominant synaptic taurine peptide. *J Neurochem* 44:752-754.
- Martens JR, Sakamoto N, Sullivan SA, Grobaski TD and Tamkun MM (2001) Isoform-specific localization of voltage-gated K<sup>+</sup> channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. *J Biol Chem* 276:8409-8414.
- Masada T, Xi G, Hua Y and Keep RF (2000) The effects of thrombin preconditioning on focal cerebral ischemia in rats. *Brain Res* 867:173-179.
- Massieu L, Montiel T, Robles G and Quesada O (2004) Brain amino acids during hyponatremia in vivo: clinical observations and experimental studies. *Neurochem Res* 29:73-81.
- Maxfield FR and Tabas I (2005) Role of cholesterol and lipid organization in disease. *Nature* 438:612-621.
- McFerrin MB and Sontheimer H (2006) A role for ion channels in glioma cell invasion. *Neuron Glia Biol* 2:39-49.
- McKinley MJ and Johnson AK (2004) The physiological regulation of thirst and fluid intake. *News Physiol Sci* 19:1-6.
- McManus ML, Churchwell KB and Strange K (1995) Regulation of cell volume in health and disease. *N Engl J Med* 333:1260-1266.
- Melton JE, Patlak CS, Pettigrew KD and Cserr HF (1987) Volume regulatory loss of Na, Cl, and K from rat brain during acute hyponatremia. *Am J Physiol* 252:F661-669.

- Miller TJ, Hanson RD and Yancey PH (2000) Developmental changes in organic osmolytes in prenatal and postnatal rat tissues. *Comp Biochem Physiol A Mol Integr Physiol* 125:45-56.
- Miyazaki T, Fujiki H, Yamamura Y, Nakamura S and Mori T (2007) Tolvaptan, an orally active vasopressin V(2)-receptor antagonist - pharmacology and clinical trials. *Cardiovasc Drug Rev* 25:1-13.
- Mongin AA, Cai Z and Kimelberg HK (1999a) Volume-dependent taurine release from cultured astrocytes requires permissive  $[Ca^{2+}]_i$  and calmodulin. *Am J Physiol* 277:C823-832.
- Mongin AA and Kimelberg HK (2002) ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. *Am J Physiol Cell Physiol* 283:C569-578.
- Mongin AA and Kimelberg HK (2005) ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple  $Ca^{2+}$ -sensitive mechanisms. *Am J Physiol Cell Physiol* 288:C204-213.
- Mongin AA and Orlov SN (2001) Mechanisms of cell volume regulation and possible nature of the cell volume sensor. *Pathophysiology* 8:77-88.
- Mongin AA, Reddi JM, Charniga C and Kimelberg HK (1999b)  $[^3H]$ taurine and D- $[^3H]$ aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. *Am J Physiol* 276:C1226-1230.
- Moorman JR and Jones LR (1998) Phospholemman: a cardiac taurine channel involved in regulation of cell volume. *Adv Exp Med Biol* 442:219-228.
- Morales-Mulia M, Pasantes-Morales H and Moran J (2000) Volume sensitive efflux of taurine in HEK293 cells overexpressing phospholemman. *Biochim Biophys Acta* 1496:252-260.
- Moran J, Maar T, Gegelashvili G, Bock E, Schousboe A and Pasantes-Morales H (1996a) Taurine deficiency and neuronal migration. *Adv Exp Med Biol* 403:519-526.
- Moran J, Maar TE and Pasantes-Morales H (1994) Impaired cell volume regulation in taurine deficient cultured astrocytes. *Neurochem Res* 19:415-420.
- Moran J, Miranda D, Pena-Segura C and Pasantes-Morales H (1997) Volume regulation in NIH/3T3 cells not expressing P-glycoprotein. II. Chloride and amino acid fluxes. *Am J Physiol* 272:C1804-1809.

- Moran J, Morales-Mulia M and Pasantes-Morales H (2001) Reduction of phospholemman expression decreases osmosensitive taurine efflux in astrocytes. *Biochim Biophys Acta* 1538:313-320.
- Moran J, Sabanero M, Meza I and Pasantes-Morales H (1996b) Changes of actin cytoskeleton during swelling and regulatory volume decrease in cultured astrocytes. *Am J Physiol* 271:C1901-1907.
- Mulligan SJ and MacVicar BA (2006) VRACs CARVe a path for novel mechanisms of communication in the CNS. *Sci STKE* 2006:pe42.
- Munger MA (2007) New agents for managing hyponatremia in hospitalized patients. *Am J Health Syst Pharm* 64:253-265.
- Murray B and Eichner ER (2004) Hyponatremia of exercise. *Curr Sports Med Rep* 3:117-118.
- Musch MW, Davis-Amaral EM, Vandeburgh HH and Goldstein L (1998) Hypotonicity stimulates translocation of ICln in neonatal rat cardiac myocytes. *Pflugers Arch* 436:415-422.
- Nagelhus EA, Lehmann A and Ottersen OP (1993) Neuronal-glia exchange of taurine during hypo-osmotic stress: a combined immunocytochemical and biochemical analysis in rat cerebellar cortex. *Neuroscience* 54:615-631.
- Nakanishi T, Yamauchi A, Nakahama H, Yamamura Y, Yamada Y, Orita Y, Fujiwara Y, Uyeda N, Takamitsu Y and Sugita M (1994) Organic osmolytes in rat renal inner medulla are modulated by vasopressin V1 and/or V2 antagonists. *Am J Physiol* 267:F146-152.
- Niemeyer MI, Cid LP, Barros LF and Sepulveda FV (2001a) Modulation of the two-pore domain acid-sensitive K<sup>+</sup> channel TASK-2 (KCNK5) by changes in cell volume. *J Biol Chem* 276:43166-43174.
- Niemeyer MI, Cid LP and Sepulveda FV (2001b) K<sup>+</sup> conductance activated during regulatory volume decrease. The channels in Ehrlich cells and their possible molecular counterpart. *Comp Biochem Physiol A Mol Integr Physiol* 130:565-575.
- Nilius B (2004) Is the volume-regulated anion channel VRAC a "water-permeable" channel? *Neurochem Res* 29:3-8.
- Nilius B and Droogmans G (2003) Amazing chloride channels: an overview. *Acta Physiol Scand* 177:119-147.
- Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V and Droogmans G (1997) Properties of volume-regulated anion channels in mammalian cells. *Prog Biophys Mol Biol* 68:69-119.

- Nilius B, Eggermont J, Voets T and Droogmans G (1996) Volume-activated Cl<sup>-</sup> channels. *Gen Pharmacol* 27:1131-1140.
- Nilius B, Voets T, Prenen J, Barth H, Aktories K, Kaibuchi K, Droogmans G and Eggermont J (1999) Role of Rho and Rho kinase in the activation of volume-regulated anion channels in bovine endothelial cells. *J Physiol* 516 (Pt 1):67-74.
- O'Connor ER and Kimelberg HK (1993) Role of calcium in astrocyte volume regulation and in the release of ions and amino acids. *J Neurosci* 13:2638-2650.
- O'Neill WC (1999) Physiological significance of volume-regulatory transporters. *Am J Physiol* 276:C995-C1011.
- O'Toole ML, Douglas PS, Laird RH and Hiller DB (1995) Fluid and electrolyte status in athletes receiving medical care at an ultradistance triathlon. *Clin J Sport Med* 5:116-122.
- Offermanns S, Laugwitz KL, Spicher K and Schultz G (1994) G proteins of the G12 family are activated via thromboxane A<sub>2</sub> and thrombin receptors in human platelets. *Proc Natl Acad Sci U S A* 91:504-508.
- Okada Y (1997) Volume expansion-sensing outward-rectifier Cl<sup>-</sup> channel: fresh start to the molecular identity and volume sensor. *Am J Physiol* 273:C755-789.
- Okada Y and Maeno E (2001) Apoptosis, cell volume regulation and volume-regulatory chloride channels. *Comp Biochem Physiol A Mol Integr Physiol* 130:377-383.
- Olson JE (1999) Osmolyte contents of cultured astrocytes grown in hypoosmotic medium. *Biochim Biophys Acta* 1453:175-179.
- Olson JE, Kreisman NR, Lim J, Hoffman-Kuczynski B, Schelble D and Leasure J (2003) Taurine and cellular volume regulation in the hippocampus. *Adv Exp Med Biol* 526:107-114.
- Ordaz B, Tuz K, Ochoa LD, Lezama R, Pena-Segura C and Franco R (2004a) Osmolytes and mechanisms involved in regulatory volume decrease under conditions of sudden or gradual osmolarity decrease. *Neurochem Res* 29:65-72.
- Ordaz B, Vaca L, Franco R and Pasantes-Morales H (2004b) Volume changes and whole cell membrane currents activated during gradual osmolarity decrease in C6 glioma cells: contribution of two types of K<sup>+</sup> channels. *Am J Physiol Cell Physiol* 286:C1399-1409.

- Palm C, Pistrosch F, Herbrig K and Gross P (2006) Vasopressin antagonists as aquaretic agents for the treatment of hyponatremia. *Am J Med* 119:S87-92.
- Papakonstanti EA, Vardaki EA and Stournaras C (2000) Actin cytoskeleton: a signaling sensor in cell volume regulation. *Cell Physiol Biochem* 10:257-264.
- Parkerson KA and Sontheimer H (2003) Contribution of chloride channels to volume regulation of cortical astrocytes. *Am J Physiol Cell Physiol* 284:C1460-1467.
- Parkerson KA and Sontheimer H (2004) Biophysical and pharmacological characterization of hypotonically activated chloride currents in cortical astrocytes. *Glia* 46:419-436.
- Pasantes-Morales H (1996) Volume regulation in brain cells: cellular and molecular mechanisms. *Metab Brain Dis* 11:187-204.
- Pasantes-Morales H, Cardin V and Tuz K (2000a) Signaling events during swelling and regulatory volume decrease. *Neurochem Res* 25:1301-1314.
- Pasantes-Morales H and Franco R (2002) Influence of protein tyrosine kinases on cell volume change-induced taurine release. *Cerebellum* 1:103-109.
- Pasantes-Morales H, Franco R, Ochoa L and Ordaz B (2002a) Osmosensitive release of neurotransmitter amino acids: relevance and mechanisms. *Neurochem Res* 27:59-65.
- Pasantes-Morales H, Franco R, Ordaz B and Ochoa LD (2002b) Mechanisms counteracting swelling in brain cells during hyponatremia. *Arch Med Res* 33:237-244.
- Pasantes-Morales H, Franco R, Torres-Marquez ME, Hernandez-Fonseca K and Ortega A (2000b) Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: contribution and mechanisms. *Cell Physiol Biochem* 10:361-370.
- Pasantes-Morales H and Morales Mulia S (2000) Influence of calcium on regulatory volume decrease: role of potassium channels. *Nephron* 86:414-427.
- Pasantes-Morales H, Sanchez Olea R, Miranda D and Moran J (1997) Volume regulation in NIH/3T3 cells not expressing P-glycoprotein. I. Regulatory volume decrease. *Am J Physiol* 272:C1798-1803.
- Patel GP and Balk RA (2007) Recognition and treatment of hyponatremia in acutely ill hospitalized patients. *Clin Ther* 29:211-229.

- Pedersen SF, Hoffmann EK and Mills JW (2001) The cytoskeleton and cell volume regulation. *Comp Biochem Physiol A Mol Integr Physiol* 130:385-399.
- Pike LJ (2006) Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res* 47:1597-1598.
- Pollock AS and Arieff AI (1980) Abnormalities of cell volume regulation and their functional consequences. *Am J Physiol* 239:F195-205.
- Roman RM, Wang Y, Lidofsky SD, Feranchak AP, Lomri N, Scharschmidt BF and Fitz JG (1997) Hepatocellular ATP-binding cassette protein expression enhances ATP release and autocrine regulation of cell volume. *J Biol Chem* 272:21970-21976.
- Romanenko VG, Rothblat GH and Levitan I (2004) Sensitivity of volume-regulated anion current to cholesterol structural analogues. *J Gen Physiol* 123:77-87.
- Rosso L, Peteri-Brunback B, Poujeol P, Hussy N and Mienville JM (2004) Vasopressin-induced taurine efflux from rat pituicytes: a potential negative feedback for hormone secretion. *J Physiol* 554:731-742.
- Rovira A, Cordoba J, Raguer N and Alonso J (2002) Magnetic resonance imaging measurement of brain edema in patients with liver disease: resolution after transplantation. *Curr Opin Neurol* 15:731-737.
- Sadoshima J, Qiu Z, Morgan JP and Izumo S (1996) Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. *Embo J* 15:5535-5546.
- Sanchez-Olea R, Moran J, Schousboe A and Pasantes-Morales H (1991) Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. *Neurosci Lett* 130:233-236.
- Sarfaraz D and Fraser CL (1999) Effects of arginine vasopressin on cell volume regulation in brain astrocyte in culture. *Am J Physiol* 276:E596-601.
- Scanlon SM, Williams DC and Schloss P (2001) Membrane cholesterol modulates serotonin transporter activity. *Biochemistry* 40:10507-10513.
- Schaffer S, Azuma J, Takahashi K and Mozaffari M (2003) Why is taurine cytoprotective? *Adv Exp Med Biol* 526:307-321.
- Schaffer S, Solodushko V and Azuma J (2000a) Taurine-deficient cardiomyopathy: role of phospholipids, calcium and osmotic stress. *Adv Exp Med Biol* 483:57-69.



- Schaffer S, Takahashi K and Azuma J (2000b) Role of osmoregulation in the actions of taurine. *Amino Acids* 19:527-546.
- Schliess F and Haussinger D (2002) The cellular hydration state: a critical determinant for cell death and survival. *Biol Chem* 383:577-583.
- Schliess F, Schreiber R and Haussinger D (1995) Activation of extracellular signal-regulated kinases Erk-1 and Erk-2 by cell swelling in H4IIE hepatoma cells. *Biochem J* 309 (Pt 1):13-17.
- Schneider SW, Yano Y, Sumpio BE, Jena BP, Geibel JP, Gekle M and Oberleithner H (1997) Rapid aldosterone-induced cell volume increase of endothelial cells measured by the atomic force microscope. *Cell Biol Int* 21:759-768.
- Schousboe A and Pasantes-Morales H (1992) Role of taurine in neural cell volume regulation. *Can J Physiol Pharmacol* 70 Suppl:S356-361.
- Setchell BP, Sanchez-Partida LG and Chairussyuhur A (1993) Epididymal constituents and related substances in the storage of spermatozoa: a review. *Reprod Fertil Dev* 5:601-612.
- Shen MR, Chou CY, Hsu KF, Hsu KS and Wu ML (1999) Modulation of volume-sensitive Cl<sup>-</sup> channels and cell volume by actin filaments and microtubules in human cervical cancer HT-3 cells. *Acta Physiol Scand* 167:215-225.
- Shen MR, Lin AC, Hsu YM, Chang TJ, Tang MJ, Alper SL, Ellory JC and Chou CY (2004) Insulin-like growth factor 1 stimulates KCl cotransport, which is necessary for invasion and proliferation of cervical cancer and ovarian cancer cells. *J Biol Chem* 279:40017-40025.
- Shennan DB, McNeillie SA and Curran DE (1994) The effect of a hyposmotic shock on amino acid efflux from lactating rat mammary tissue: stimulation of taurine and glycine efflux via a pathway distinct from anion exchange and volume-activated anion channels. *Exp Physiol* 79:797-808.
- Song D, O'Regan MH and Phillis JW (1998) Protein kinase inhibitors attenuate cardiac swelling-induced amino acid release in the rat. *J Pharm Pharmacol* 50:1280-1286.
- Strange K (2004) Cellular volume homeostasis. *Adv Physiol Educ* 28:155-159.
- Strange K, Emma F and Jackson PS (1996) Cellular and molecular physiology of volume-sensitive anion channels. *Am J Physiol* 270:C711-730.
- Strange K and Jackson PS (1995) Swelling-activated organic osmolyte efflux: a new role for anion channels. *Kidney Int* 48:994-1003.

- Striggow F, Riek-Burchardt M, Kiesel A, Schmidt W, Henrich-Noack P, Breder J, Krug M, Reymann KG and Reiser G (2001) Four different types of protease-activated receptors are widely expressed in the brain and are up-regulated in hippocampus by severe ischemia. *Eur J Neurosci* 14:595-608.
- Striggow F, Riek M, Breder J, Henrich-Noack P, Reymann KG and Reiser G (2000) The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations. *Proc Natl Acad Sci U S A* 97:2264-2269.
- Stutzin A, Torres R, Oporto M, Pacheco P, Eguiguren AL, Cid LP and Sepulveda FV (1999) Separate taurine and chloride efflux pathways activated during regulatory volume decrease. *Am J Physiol* 277:C392-402.
- Sullivan R, Kunze DL and Kroll MH (1996) Thrombin receptors activate potassium and chloride channels. *Blood* 87:648-656.
- Suzuki M, Morita T and Iwamoto T (2006) Diversity of Cl(-) channels. *Cell Mol Life Sci* 63:12-24.
- Szucs G, Heinke S, Droogmans G and Nilius B (1996) Activation of the volume-sensitive chloride current in vascular endothelial cells requires a permissive intracellular Ca<sup>2+</sup> concentration. *Pflugers Arch* 431:467-469.
- Tang X, Batty IH and Downes CP (2002) Muscarinic receptors mediate phospholipase C-dependent activation of protein kinase B via Ca<sup>2+</sup>, ErbB3, and phosphoinositide 3-kinase in 1321N1 astrocytoma cells. *J Biol Chem* 277:338-344.
- Tilly BC, Edixhoven MJ, Tertoolen LG, Morii N, Saitoh Y, Narumiya S and de Jonge HR (1996) Activation of the osmo-sensitive chloride conductance involves P21rho and is accompanied by a transient reorganization of the F-actin cytoskeleton. *Mol Biol Cell* 7:1419-1427.
- Tilly BC, van den Berghe N, Tertoolen LG, Edixhoven MJ and de Jonge HR (1993) Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. *J Biol Chem* 268:19919-19922.
- Toman RE and Spiegel S (2002) Lysophospholipid receptors in the nervous system. *Neurochem Res* 27:619-627.
- Trejo J (2003) Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. *J Pharmacol Exp Ther* 307:437-442.

- Trimarchi JR, Liu L, Smith PJ and Keefe DL (2002) Apoptosis recruits two-pore domain potassium channels used for homeostatic volume regulation. *Am J Physiol Cell Physiol* 282:C588-594.
- Trouet D, Hermans D, Droogmans G, Nilius B and Eggermont J (2001) Inhibition of volume-regulated anion channels by dominant-negative caveolin-1. *Biochem Biophys Res Commun* 284:461-465.
- Trouet D, Nilius B, Jacobs A, Remacle C, Droogmans G and Eggermont J (1999) Caveolin-1 modulates the activity of the volume-regulated chloride channel. *J Physiol* 520 Pt 1:113-119.
- Ullrich N, Caplanusi A, Brone B, Hermans D, Lariviere E, Nilius B, Van Driessche W and Eggermont J (2006) Stimulation by caveolin-1 of the hypotonicity-induced release of taurine and ATP at basolateral, but not apical, membrane of Caco-2 cells. *Am J Physiol Cell Physiol* 290:C1287-1296.
- Valverde MA, Bond TD, Hardy SP, Taylor JC, Higgins CF, Altamirano J and Alvarez-Leefmans FJ (1996) The multidrug resistance P-glycoprotein modulates cell regulatory volume decrease. *Embo J* 15:4460-4468.
- Vanoye CG, Altenberg GA and Reuss L (1997) P-glycoprotein is not a swelling-activated Cl<sup>-</sup> channel; possible role as a Cl<sup>-</sup> channel regulator. *J Physiol* 502 (Pt 2):249-258.
- Vaughan PJ, Pike CJ, Cotman CW and Cunningham DD (1995) Thrombin receptor activation protects neurons and astrocytes from cell death produced by environmental insults. *J Neurosci* 15:5389-5401.
- Verbalis JG (1993) Hyponatremia: epidemiology, pathophysiology, and therapy. *Curr Opin Nephrol Hypertens* 2:636-652.
- Verbalis JG and Gullans SR (1993) Rapid correction of hyponatremia produces differential effects on brain osmolyte and electrolyte reaccumulation in rats. *Brain Res* 606:19-27.
- Verhoeven A, Musch W and Decaux G (2005) Treatment of the polydipsia-hyponatremia syndrome with urea. *J Clin Psychiatry* 66:1372-1375.
- Verkman AS and Mitra AK (2000) Structure and function of aquaporin water channels. *Am J Physiol Renal Physiol* 278:F13-28.
- Videen JS, Michaelis T, Pinto P and Ross BD (1995) Human cerebral osmolytes during chronic hyponatremia. A proton magnetic resonance spectroscopy study. *J Clin Invest* 95:788-793.

- Villullas IR, Smith AJ, Heavens RP and Simpson PB (2003) Characterisation of a sphingosine 1-phosphate-activated Ca<sup>2+</sup> signalling pathway in human neuroblastoma cells. *J Neurosci Res* 73:215-226.
- Voets T, Droogmans G and Nilius B (1997) Modulation of voltage-dependent properties of a swelling-activated Cl<sup>-</sup> current. *J Gen Physiol* 110:313-325.
- Voets T, Droogmans G, Raskin G, Eggermont J and Nilius B (1999) Reduced intracellular ionic strength as the initial trigger for activation of endothelial volume-regulated anion channels. *Proc Natl Acad Sci U S A* 96:5298-5303.
- Voets T, Manolopoulos V, Eggermont J, Ellory C, Droogmans G and Nilius B (1998) Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. *J Physiol* 506 (Pt 2):341-352.
- Wade JV, Olson JP, Samson FE, Nelson SR and Pazdernik TL (1988) A possible role for taurine in osmoregulation within the brain. *J Neurochem* 51:740-745.
- Wang H and Reiser G (2003) Thrombin signaling in the brain: the role of protease-activated receptors. *Biol Chem* 384:193-202.
- Wang H, Ubl JJ and Reiser G (2002) Four subtypes of protease-activated receptors, co-expressed in rat astrocytes, evoke different physiological signaling. *Glia* 37:53-63.
- Wang L, Chen L and Jacob TJ (2000) The role of ClC-3 in volume-activated chloride currents and volume regulation in bovine epithelial cells demonstrated by antisense inhibition. *J Physiol* 524 Pt 1:63-75.
- Wehner F, Shimizu T, Sabirov R and Okada Y (2003) Hypertonic activation of a non-selective cation conductance in HeLa cells and its contribution to cell volume regulation. *FEBS Lett* 551:20-24.
- Weylandt KH, Valverde MA, Nobles M, Raguz S, Amey JS, Diaz M, Nastrucci C, Higgins CF and Sardini A (2001) Human ClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. *J Biol Chem* 276:17461-17467.
- Wise BL and Chater N (1962) The value of hypertonic mannitol solution in decreasing brain mass and lowering cerebro-spinal-fluid pressure. *J Neurosurg* 19:1038-1043.
- Wood JM (1999) Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol Mol Biol Rev* 63:230-262.

- Xi G, Keep RF, Hua Y, Xiang J and Hoff JT (1999) Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke* 30:1247-1255.
- Xi G, Reiser G and Keep RF (2003) The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J Neurochem* 84:3-9.
- Xu X and London E (2000) The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry* 39:843-849.
- Xu YJ, Saini HK, Zhang M, Elimban V and Dhalla NS (2006b) MAPK activation and apoptotic alterations in hearts subjected to calcium paradox are attenuated by taurine. *Cardiovasc Res* 72:163-174.
- Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* 208:2819-2830.
- Yancey PH, Clark ME, Hand SC, Bowlus RD and Somero GN (1982) Living with water stress: evolution of osmolyte systems. *Science* 217:1214-1222.
- Yoshida Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K and Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol* 38:1095-1102.
- Zidovetzki R and Levitan I (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* 1768:1311-1324.
- Zipp F, Waiczies S, Aktas O, Neuhaus O, Hemmer B, Schraven B, Nitsch R and Hartung HP (2007) Impact of HMG-CoA reductase inhibition on brain pathology. *Trends Pharmacol Sci* 28:342-349.

## CHAPTER 2

### SUBNANOMOLAR CONCENTRATIONS OF THROMBIN ENHANCE THE VOLUME-SENSITIVE EFFLUX OF TAURINE FROM HUMAN 1321N1 ASTROCYTOMA CELLS

#### Summary

The ability of subnanomolar concentrations of thrombin to protect both neurons and glia from ischemia and other metabolic insults has recently been reported. In this study, we demonstrate an additional neuroprotective property of thrombin; its ability to promote the release of the organic osmolyte, taurine, in response to hypoosmotic stress. Incubation of human 1321N1 astrocytoma cells with hypoosmolar buffers (320-227 mOsM) resulted in a time-dependent release of taurine. Inclusion of thrombin ( $EC_{50}=60$  pM), resulted in a marked increase in taurine efflux which, although evident under isotonic conditions (340 mOsM), was maximal at an osmolarity of 270 mOsM (3-4 fold stimulation). Thrombin-stimulated taurine efflux was dependent upon its protease activity and could be mimicked by addition of the peptide SFLLRN, a Proteinase Activated Receptor-1 (PAR-1) subtype specific ligand. Inclusion of anion channel blockers known to inhibit the volume-sensitive organic osmolyte anion channel attenuated thrombin-stimulated taurine release. Depletion of intracellular  $Ca^{2+}$  with either 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) or thapsigargin, or alternatively, inhibition of protein kinase C (PKC) with bisindolylmaleimide or

chelerythrine, resulted in a 30-50% inhibition of thrombin-stimulated taurine efflux. Under conditions in which intracellular  $\text{Ca}^{2+}$  was depleted and PKC activity inhibited, thrombin-stimulated taurine efflux was reduced by >85%. The results indicate that activation of PAR-1 receptors by thrombin facilitates the ability of 1321N1 astrocytoma cells to release osmolytes in response to a reduction in osmolarity via a mechanism that is dependent on intracellular  $\text{Ca}^{2+}$  and PKC activity.

### **Introduction**

Recent evidence indicates that in addition to its role in platelet aggregation and tissue repair, thrombin, a serine protease generated from prothrombin, regulates numerous physiological and pathological responses including development, inflammation, atherogenesis, stroke and Alzheimer's disease (Gingrich and Traynelis, 2000; Xi et al., 2003; Ossovaskaya and Bunnett, 2004; Suo et al., 2004). Thrombin may also be produced in the brain following cerebral hemorrhage or disruption of the blood brain barrier. It mediates its cellular functions through a family of receptors known as Proteinase Activated Receptors (PARs). PARs belong to the superfamily of G-protein coupled receptors (GPCRs) which operate via  $G_q$ ,  $G_i$  or  $G_{12/13}$  families of G-proteins and currently four members of the group (PAR1, PAR2, PAR3 and PAR4) have been identified. PARs have a unique activation mechanism. They are activated by an irreversible proteolytic cleavage of the receptor's extracellular N-terminus, thus unmasking a new N-terminus that functions as a "tethered peptide ligand," which

then binds intra-molecularly to the receptors and initiates intracellular signal transduction (Macfarlane et al., 2001; Hollenberg and Compton, 2002; Trejo, 2003; Wang and Reiser, 2003).

All four PARs are expressed abundantly in the central nervous system (Striggow et al., 2001); however, their function is still unclear. Previous studies indicate biphasic and dose-dependent actions of thrombin on astrocytes. Low concentrations of thrombin (50 pM to 100 nM) mediate neuroprotection against ischemia and environmental insults such as oxidative stress, hypoglycemia, hypoxia and growth supplement deprivation. High concentrations of thrombin however, can cause degeneration and cell death (Vaughan et al., 1995; Striggow et al., 2000; Jiang et al., 2002). *In vivo*, pretreatment of the brain with a low dose of thrombin attenuates brain injury induced by cerebral hemorrhage or trauma although high dose thrombin infusion can cause astrogliosis (Xi et al., 1999; Masada et al., 2000). It has also been demonstrated that PAR1 and PAR3 receptors are up-regulated in various regions of the brain after environmental insults (Xi et al., 2003). Recently, PAR1 and PAR3 were shown to mediate anti-apoptotic signaling by activated protein C in neurons (Guo et al., 2004). Although these studies indicate the potential importance of low concentration of thrombin for the development of new therapeutic strategies to treat neurodegenerative disorders, the molecular mechanism(s) underlying neuroprotection remain to be established.

A neuroprotective mechanism utilized by both neurons and glia is that of volume regulation following exposure of the cells to osmotic insult. In response



to hypotonic stress, cells swell with a magnitude proportional to the reduction in osmolarity. This rapid increase in volume is transient and followed by a recovery process of regulatory volume decrease (RVD), during which intracellular osmolytes ( $K^+$ ,  $Cl^-$  and organic osmolytes) are extruded and cell volume normalized following the exit of intracellular water (McManus et al., 1995). Of the organic osmolytes utilized by cells, taurine is ideally suited because of its abundance, water solubility and metabolic inertness (Lambert, 2004). Swelling of neural cells due to fluctuations in osmolarity is very common in elderly, infants and during pregnancy. It can be derived from excessive water intake, such as occurs in athletes and psychotic polydipsia, or alternatively in conditions such as glucocorticoid deficiency, hypothyroidism, use of thiazide diuretics, and renal or hepatic failure (Kimmelberg, 2000; Pasantes-Morales et al., 2000, 2002). The principal cell type involved in volume regulation within the central nervous system is the astrocyte, since these cells comprise up to 90% of cell number within the brain. The importance of taurine release in RVD is indicated from studies in which taurine-deficient astrocytes were shown to exhibit a less efficient volume recovery in comparison to control cells (Moran et al., 1994). The extrusion of taurine occurs predominantly via a channel known as Volume Sensitive Organic Osmolyte Anion Channel (VSOAC). VSOAC is a chloride channel, impermeable to cations (for reviews, see Nilius et al., 1997; Lang et al., 1998; Nilius and Droogmans, 2003). Taurine efflux, as well as RVD, can be blocked by classical non-selective  $Cl^-$  channel inhibitors, such as 1,9-dideoxyforskolin (DDF), 5-nitro-

2-(3-phenylpropylamine)benzoic acid (NPPB), 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS) and 4-acetamido-4-isothiocyano-2,2'-disulfonic stilbene (SITS).

In the present study, we demonstrate that subnanomolar concentrations of thrombin, operating primarily via a PAR-1 receptor, facilitate the volume-sensitive efflux of taurine from human astrocytoma cells. Thrombin-stimulated taurine efflux is mediated via a VSOAC channel and intracellular  $Ca^{2+}$  and protein kinase C (PKC) are implicated in the mechanism of osmolyte release. A preliminary account of part of this study has previously been reported (Cheema et al., 2005).

## Methods

**Materials.** [1,2- $^3H$ ]Taurine (1.15 TBq/ml) was obtained from Amersham Biosciences (Piscataway, NJ). Thrombin, DIDS, NPPB, SITS and 4 $\alpha$ -phorbol 12,13-didecanoate were purchased from Sigma-Aldrich (St. Louis, MO). 1,9-Dideoxyforskolin, PMA, ionomycin, KN-93, PD 98059 and wortmannin were obtained from Calbiochem (San Diego, CA). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). Thrombin receptor activating peptides, SFLLRN, TFLLRN, TFRGAP, GYPGKF were obtained from BaChem (Torrance, CA). Guanidineethyl sulfonate was obtained from Toronto Chemicals (Toronto, ON). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (Franklin Lakes, NJ).

Universol was obtained from ICN biomedical (Uroora, OH). Dowex-1 resin 100-200 mesh; x 8 formate) was obtained from Bio-Rad (Hercules, CA).

### **Cell culture conditions.**

1321N1 astrocytoma cells (passages 5-21) were grown in tissue culture flasks (75 cm<sup>2</sup>/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330 - 340mOsM. Cells were grown at 37°C in a humidified atmosphere containing 10% CO<sub>2</sub>. The medium was aspirated and the cells detached from the flask with a trypsin-versene mixture (Biowhittaker, MD). Cells were then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates for 3-5 days. Experiments were routinely conducted on cells that had reached 50–90% confluency.

### **Preparation of primary astrocyte cultures**

Neonatal cultures of rat astrocytes were prepared from 2-day-old rats (Sprague Dawley) essentially according to the method previously described (Jiang et al., 2002).

### **Measurement of taurine efflux.**

Osmolyte efflux from 1321N1 astrocytoma cells was monitored essentially as previously described (Loveday et al. 2003, Heacock et al., 2004). In brief, cells were pre-labeled overnight with 18.5 KBq/ml of [<sup>3</sup>H]taurine at 37°C. Under these

conditions, approximately 10-20% of the added radiolabel was taken up into the cells. Uptake of radiolabel into 1321N1 cells was time-dependent ( $t_{1/2} \sim 7$ h), temperature sensitive (inhibited >98% by lowering the temperature to 4°C) and was inhibited by 75-80% by inclusion of a 500  $\mu$ M guanidineethyl sulfonate, an inhibitor of the taurine uptake transporter (Lambert, 2004). After prelabeling, the cells were washed with 2 x 2 ml of isotonic Buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl<sub>2</sub>, 3.6 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub> and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 340 mOsM). Cells were then allowed to incubate in 2ml of hypotonic buffer A (320-200 mOsM; rendered hypotonic by a reduction in NaCl concentration) in the absence or presence of thrombin. In some experiments, buffer A was made hypertonic (370 mOsM) by the addition of NaCl. Osmolarities of buffer A were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots (200  $\mu$ l) of the extracellular medium were removed and radioactivity determined after the addition of 5ml Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 x 1 ml of ice-cold 6% (wt/vol) trichloroacetic acid. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released in the extracellular media as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay (Novak et al., 1999). "Basal" release of taurine is defined as that which occurs at a specified osmolarity in the absence of thrombin.

### **Measurement of Phosphoinositide Turnover.**

To monitor phosphoinositide turnover, 1321N1 cells that had been prelabeled with 148 KBq/ml of [<sup>3</sup>H]inositol for 96 h were incubated in hypotonic buffer A (270 mOsm) that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher, 1990).

### **Measurement of Cytoplasmic Calcium Concentration.**

Cytoplasmic free calcium concentrations,  $[Ca^{2+}]_i$ , were determined in suspensions of 1321N1 astrocytoma cells after preloading cells with the  $Ca^{2+}$  indicator, fura-2 AM (Molecular Probes, OR), as previously described (Fisher et al., 1989). The fluorometer used was a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD).

### **Data analysis.**

Experiments were performed in triplicate and repeated at least three times. Values quoted are given as means  $\pm$  SEM for the number (n) of independent experiments indicated. A two-tailed Student's t-test (paired) was used to evaluate differences between two experimental groups (level of significance,  $p < 0.05$ ). One-way or repeated measures Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups.  $EC_{50}$  values were obtained using Prism 3.03 (GraphPad Software, Inc. San Diego, CA).

## Results

### **Osmosensitive efflux of taurine from 1321N1 astrocytoma cells is enhanced**

**by the addition of thrombin.** When 1321N1 astrocytoma cells that had been prelabeled with [<sup>3</sup>H]taurine were exposed to hypotonic buffer (270 mOsM), there was a time-dependent release of the radiolabeled amino acid from the cells (Fig. 2.1A). The initial rate of release monitored over the first 5 min was greater than that observed following prolonged incubations. Inclusion of thrombin (0.25U/ml equivalent to 1.25 nM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of response by approximately 3-fold over basal (basal release being that monitored in the absence of thrombin; Fig. 2.1A). If 1321N1 cells were first exposed to hypotonic buffer for 10 min and then thrombin added (i.e. after the initial rapid phase of taurine efflux), a significant stimulation of taurine release was still observed (Fig. 2.1B). As a result of these observations, taurine efflux was routinely monitored after a 20 min incubation in subsequent experiments. The addition of thrombin resulted in a concentration-dependent stimulation of taurine efflux with a maximum effect observed at 1.25 nM (Fig. 2.2). The EC<sub>50</sub> value for thrombin-stimulated taurine release from 1321N1 astrocytoma was 0.06 nM.

### **Thrombin enhances osmosensitive taurine release via the PAR-1 receptor.**

To determine whether the ability of thrombin to enhance taurine release was mediated via its protease action at a receptor, two series of experiments were performed. In the first, thrombin was pretreated with 4 μM D-Phe-Pro-Arg

Chloromethyl Ketone (PPACK), a protease inhibitor. Although PPACK had no effect on basal taurine release, it essentially abolished the ability of thrombin to enhance taurine release (Fig. 2.3A). In a second series of experiments, the ability of three synthetic Proteinase-Activated Receptor (PAR) peptides to enhance taurine release was evaluated. Addition of 100  $\mu$ M SFFLRN, a synthetic peptide specific for PAR-1 subtype, significantly increased taurine release over basal (275% of basal; Fig. 2.3B). This enhancement, which was also observed with 100  $\mu$ M TFFLRN-an additional PAR-1-specific peptide, was similar in magnitude to that observed for thrombin. Although inclusion of 500  $\mu$ M PAR-3 specific peptide (TFRGAP) also increased taurine release, the effect was not significant and was markedly less than that of the PAR-1 agonist. In contrast, the addition of the PAR-4 peptide, GYPGKF, did not significantly increase taurine release over basal. Taken collectively, these data indicate that the ability of thrombin to enhance taurine release in 1321N1 astrocytoma is mediated primarily by the PAR-1 receptor subtype. The ability of thrombin to stimulate taurine release under hypotonic conditions was also observed for primary cultures of rat astrocytes, although the effect was less marked (148% of basal; Fig. 2.3C) than that observed for 1321N1 astrocytoma cells.

**Thrombin enhances the volume-sensitive efflux of taurine from 1321N1 astrocytoma cells as osmolarity decreases.** The ability of thrombin to potentiate the release of taurine at different osmolarities was examined. Both basal and thrombin-stimulated release of taurine was monitored under conditions

of isotonicity (340 mOsM: defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells are grown), mild- severe hypotonicity (320-227 mOsM) or mild hypertonicity (370 mOsM). In the series of experiments conducted, the basal release of taurine was not significantly enhanced until the osmolarity of the buffer had been reduced to 227 mOsM. In contrast, the addition of thrombin resulted in a significant increase in taurine efflux (279% of basal) even under isotonic conditions (Fig. 2.4). Moreover, as the osmolarity of the buffer was reduced, the ability of thrombin to enhance taurine efflux over the basal component was further increased. The maximum enhancement of taurine efflux was observed at an osmolarity of 270 mOsM (442 % of basal). In contrast, when cells were exposed to mildly hypertonic buffer A (370 mOsM), the addition of thrombin did not significantly enhance taurine release. As a result of these findings, an osmolarity of 270 mOsM was chosen for all subsequent experiments.

**Taurine efflux from 1321N1 astrocytoma is mediated via a VSOAC.** To determine whether thrombin-stimulated taurine release occurred via a VSOAC, the ability of four anion channel inhibitors, all of which are putative blockers of the VSOAC channel, to inhibit the basal and thrombin-stimulated efflux of taurine was examined. All four anion channel inhibitors resulted in a significant inhibition of both basal- and thrombin-stimulated taurine release (Fig. 2.5). At a concentration of 100  $\mu$ M, dideoxyforskolin and NPPB were more effective at inhibiting taurine release than either of the stilbene derivatives, DIDS and SITS (67-94% inhibition vs. 40-64% inhibition) at concentrations of 100  $\mu$ M and 500



$\mu\text{M}$ , respectively. Higher concentrations of the latter agents could not be tested as they resulted in detachment of cells from the dishes.

**Thrombin-stimulated taurine release is unaffected by pertussis toxin or inclusion of inhibitors of phosphatidylinositol 3-kinase, mitogen-activated protein kinase or  $\text{Ca}^{2+}$  calmodulin dependent protein kinase II signaling pathways.** Since the PAR-1 subtype is known to couple to the pertussis toxin-sensitive  $G_i$  subfamily of heterotrimeric G proteins (Coughlin, 2000), we tested the ability of pertussis toxin to inhibit thrombin-stimulated taurine release. Overnight pretreatment of the cells with pertussis toxin (60 ng/ml) resulted in a small reduction in basal release of taurine ( $68 \pm 9\%$  of control,  $n=6$ ), whereas thrombin-stimulated taurine efflux was unaffected ( $387 \pm 54\%$  vs.  $509 \pm 29\%$  of basal for untreated- and pertussis toxin-treated cells, respectively,  $n=6$ ).

To examine the possibility that other known mediators of PAR-1 activation were involved in the thrombin-induced taurine release, cells were incubated with wortmannin (100 nM), a phosphatidylinositol 3-kinase inhibitor or PD 98059 (50  $\mu\text{M}$ ), mitogen activated protein (MAP) kinase inhibitor (Wang et al., 2002), and KN-93 (10  $\mu\text{M}$ ), a  $\text{Ca}^{2+}$  CaMK II inhibitor. No significant effect on either basal or thrombin-stimulated taurine release was observed for any of these inhibitors (data not shown).

**Taurine release from 1321N1 astrocytoma cells is enhanced following a rise in the concentration of intracellular calcium or activation of protein kinase**

**C.** Activation of thrombin receptors on 1321N1 astrocytoma cell has also been reported to elicit an increase in the activity of phospholipase C (PLC) mediated via  $G_q$ , with a concomitant rise in the concentration of cytoplasmic calcium,  $[Ca^{2+}]_i$ , and activation of PKC (Jones et al., 1989). In agreement with these previous observations, the addition of thrombin to 1321N1 cells (incubated in hypotonic buffer A) resulted in a small, but significant, increase in the release of inositol phosphates (146 and 174% of basal after 5 or 10 min respectively; Fig. 2.6A). Thrombin addition also elicited a rise in  $[Ca^{2+}]_i$  ( $592 \pm 78$  nM vs  $196 \pm 23$  nM for basal,  $n=13$ ), which was markedly attenuated when extracellular  $Ca^{2+}$  was omitted ( $69 \pm 4\%$  inhibition,  $n=14$ ; Fig. 2.6B). In the absence of extracellular  $Ca^{2+}$ , the depletion of intracellular  $Ca^{2+}$  stores with 5  $\mu$ M thapsigargin further reduced the ability of thrombin to increase  $[Ca^{2+}]_i$  ( $89 \pm 2\%$  inhibition,  $n = 8$ ).

A rise in  $[Ca^{2+}]_i$ , mediated by the addition of 1  $\mu$ M ionomycin (which facilitates both the influx of extracellular  $Ca^{2+}$  and the release of  $Ca^{2+}$  from intracellular stores), partially mimicked the ability of thrombin to enhance taurine efflux from the astrocytoma cells (165% of basal; Fig. 2.7A). A similar significant increase in taurine release was observed following the addition of PMA, a PKC agonist (170% of basal; Fig. 2.7A) whereas its inactive analog, 4- $\alpha$ -phorbol 12,13-didecanoate, was without effect (data not shown). When both PMA and ionomycin were added to the hypotonically treated cells, their effect on taurine release was additive (219% of basal). However, thrombin-stimulated taurine efflux was not further enhanced by the presence of either PMA or ionomycin (Fig.2.7B)

To determine the role, if any, played by  $\text{Ca}^{2+}$  in basal- and thrombin-stimulated taurine efflux, taurine release was monitored under conditions in which extracellular- and/or intracellular  $\text{Ca}^{2+}$  had been depleted. Removal of extracellular  $\text{Ca}^{2+}$  had little or no effect on either basal- or thrombin-stimulated taurine efflux (Fig. 2.8A). In contrast, chelation of intracellular  $\text{Ca}^{2+}$  with BAPTA-AM significantly reduced the extent of the thrombin-stimulated taurine efflux (56% inhibition) whereas basal efflux was unaffected. To further examine the role of intracellular  $\text{Ca}^{2+}$  in osmolyte release, the cells were preincubated with 5  $\mu\text{M}$  thapsigargin (in the absence of extracellular  $\text{Ca}^{2+}$ ) to discharge the intracellular  $\text{Ca}^{2+}$  pools. Under these conditions, the ability of thrombin to stimulate taurine efflux was reduced by 52% (Fig. 2.8B).

To test the involvement of PKC in thrombin-stimulated taurine efflux, cells were preincubated with either 1  $\mu\text{M}$  BIM or 10  $\mu\text{M}$  chelerythrine. Although BIM slightly decreased the basal taurine efflux, both BIM and chelerythrine significantly attenuated thrombin-stimulated taurine release (30% inhibition; Fig. 2.9A). Down-regulation of PKC following overnight incubation of the cells with 100 nM PMA also resulted in reduction in an inhibition of thrombin-stimulated taurine efflux ( $54 \pm 6\%$  vs control cells,  $n=3$ ). The combination of inhibition of PKC with 10  $\mu\text{M}$  chelerythrine, along with depletion of intracellular  $\text{Ca}^{2+}$  with 5  $\mu\text{M}$  thapsigargin, resulted in an 87% inhibition of thrombin-stimulated taurine release (Fig. 2.9B).

## Discussion

Subnanomolar concentrations of thrombin have been demonstrated to protect both neurons and astrocytes against metabolic insults such as hypoglycemia, ischemia or oxidative stress (Vaughan et al., 1995; Striggow et al., 2000). In the present study, we demonstrate that similarly low concentrations of thrombin also markedly increase the release of taurine, an organic osmolyte, from human 1321N1 astrocytoma cells following a mild hypoosmotic insult. The ability of thrombin to enhance taurine release, which is dependent on its protease activity, occurs rapidly (within 1-2 min of addition) and can be observed regardless of whether thrombin is added prior to or after the initiation of taurine release (Figs. 2.1A and B). The concentrations of thrombin required to enhance taurine release ( $EC_{50}$  = 60 pM) is similar to those previously demonstrated to provide neuroprotection (Fig. 2.2). Based upon the ability of the receptor-specific peptides SFLLRN and TFLLRN to fully mimic the ability of thrombin to enhance taurine release (and the relative ineffectiveness of PAR-3- and PAR-4-specific peptides), it appears that the PAR-1 receptor subtype is primarily responsible for thrombin-stimulated osmolyte release (Fig. 2.3B). Although an astrocytoma cell line was primarily utilized for the present study, we observed that the addition of thrombin to primary cultures of rat brain astrocytes incubated under hypoosmotic conditions also resulted in a stimulation of taurine release above basal (Fig. 2.3C). Although the ability of thrombin to stimulate osmolyte release has previously been reported for myoblasts (Manolopoulos et al. 1997), to the best of our knowledge, the present results are the first to indicate a similar role for

thrombin in neural tissues and to identify the receptor subtype involved. Thus one of the *in vivo* functions of thrombin in the brain, whether synthesized within the CNS or resulting from cerebral hemorrhage or a compromised blood-brain barrier, may be that of osmoregulation.

The pharmacological profile of inhibition of both basal- and thrombin-stimulated taurine efflux from 1321N1 astrocytoma cells by anion channel inhibitors is consistent with the involvement of a VSOAC in osmolyte release. Thus, taurine efflux was significantly inhibited by DIDS, SITS, NPPB and DDF, all of which are purported to be inhibitors of volume-sensitive anion channels (Nilius et al., 1997). However, as previously observed for the volume-dependent efflux of taurine from SH-SY5Y neuroblastoma cells (Heacock et al., 2004), DDF and NPPB are markedly more potent inhibitors of osmolyte release from the astrocytoma cells than either of the two stilbene derivatives, DIDS and SITS.

One notable feature of thrombin-stimulated taurine efflux is that the protease is able to substantially enhance osmolyte release (279% of basal) even under isotonic conditions (340 mOsm; Fig. 2.4). This result suggests that even though the VSOAC primarily responds to a reduction of osmolarity, the channel is partially open under conditions of isotonicity, but not under hypertonic conditions (370 mOsm). The ability of receptor activation to enhance osmolyte release under isotonic conditions has been observed previously. For example, ATP-induced D-aspartate release from astrocytes and muscarinic cholinergic receptor (mAChR)-mediated taurine efflux from neuroblastoma cells can be observed under isotonic conditions (Mongin and Kimelberg, 2002; Heacock et al., 2004).

In contrast, the mAChR-stimulated release of *myo*-inositol, another quantitatively major organic osmolyte, was not observed under isotonic conditions (Loveday et al., 2003). In this context, it should be noted that the ability of organic osmolytes to permeate VSOAC is dependent upon their molecular dimensions. Since the minimum diameter of the pore channel of the VSOAC is reported to be between 5.4 and 8.0 Å (McManus et al., 1995; Nilius et al., 1997), osmolytes such as glutamate, D-aspartate or taurine will readily exit the cells, whereas *myo*-inositol, whose molecular dimensions are close to the pore diameter, is released less readily. Although activation of PAR-1 receptors on astrocytoma cells can enhance taurine release under isotonic conditions, its effects become even more pronounced when the osmolarity is reduced by 5-20% – conditions under which the basal release of taurine is only minimally increased (Fig. 2.4). These results are consistent with the concept that PAR-1 activation facilitates the ability of the cells to release osmolytes (and, by inference, to regulate their volume) under conditions of very limited reductions in osmolarity, i.e. those that might be expected to pertain to physiological or pathological conditions *in vivo*.

Although PAR-1 receptors are pleiotropic and can couple to multiple GTP-binding proteins thereby activating a diverse array of signaling pathways (Coughlin, 2000; Trejo, 2003), our data suggest that  $\text{Ca}^{2+}$  and PKC play the major roles in facilitation of taurine release. The evidence for this is as follows. First, the ability of thrombin to enhance taurine release could be mimicked, in part at least, by the addition of the  $\text{Ca}^{2+}$  ionophore, ionomycin. Second, the mobilization of an intracellular pool of  $\text{Ca}^{2+}$  appears to be required since either

chelation of intracellular  $\text{Ca}^{2+}$  with BAPTA, or discharge of the pool with thapsigargin, resulted in a 52-56% inhibition of thrombin-stimulated taurine efflux. In contrast, removal of extracellular  $\text{Ca}^{2+}$  had no effect on the magnitude of efflux. The reliance of thrombin-stimulated taurine release on intracellular (rather than extracellular) stores of  $\text{Ca}^{2+}$  contrasts with the  $\text{Ca}^{2+}$  signals generated in fura-2 loaded cells upon thrombin addition, which depend on both sources of  $\text{Ca}^{2+}$  (Fig. 2.6B). This result suggests that (1) thrombin-stimulated taurine release may require a rise in  $[\text{Ca}^{2+}]_i$ , (2) the magnitude of osmolyte release is not directly proportional to that of the  $\text{Ca}^{2+}$  signal. Moreover, it appears that a significant fraction of thrombin-stimulated osmolyte release can still occur in the absence of  $\text{Ca}^{2+}$  (see Fig. 2.8B). Although thrombin addition results in an activation of PLC (Fig. 2.6A) and a rise in  $[\text{Ca}^{2+}]_i$  in these cells, an obligatory link between this pathway and osmolyte release is yet to be established because of the absence of a specific inhibitor of PLC (see Loveday et al., 2003).

A role for PKC is indicated from the ability of PMA, when added alone, to stimulate taurine efflux and from the observation that either BIM or chelerythrine, two inhibitors of PKC, can inhibit thrombin-stimulated taurine release. Further indication of the involvement of PKC was obtained from experiments in which down-regulation of the enzyme, following an overnight incubation of the cells with PMA, resulted in an attenuation of thrombin-stimulated taurine efflux. Under conditions in which both intracellular  $\text{Ca}^{2+}$  is depleted and PKC inhibited, thrombin's ability to stimulate osmolyte release was inhibited by 87%. In contrast to the  $\text{Ca}^{2+}$ - and PKC-dependence of thrombin-stimulated taurine efflux, the

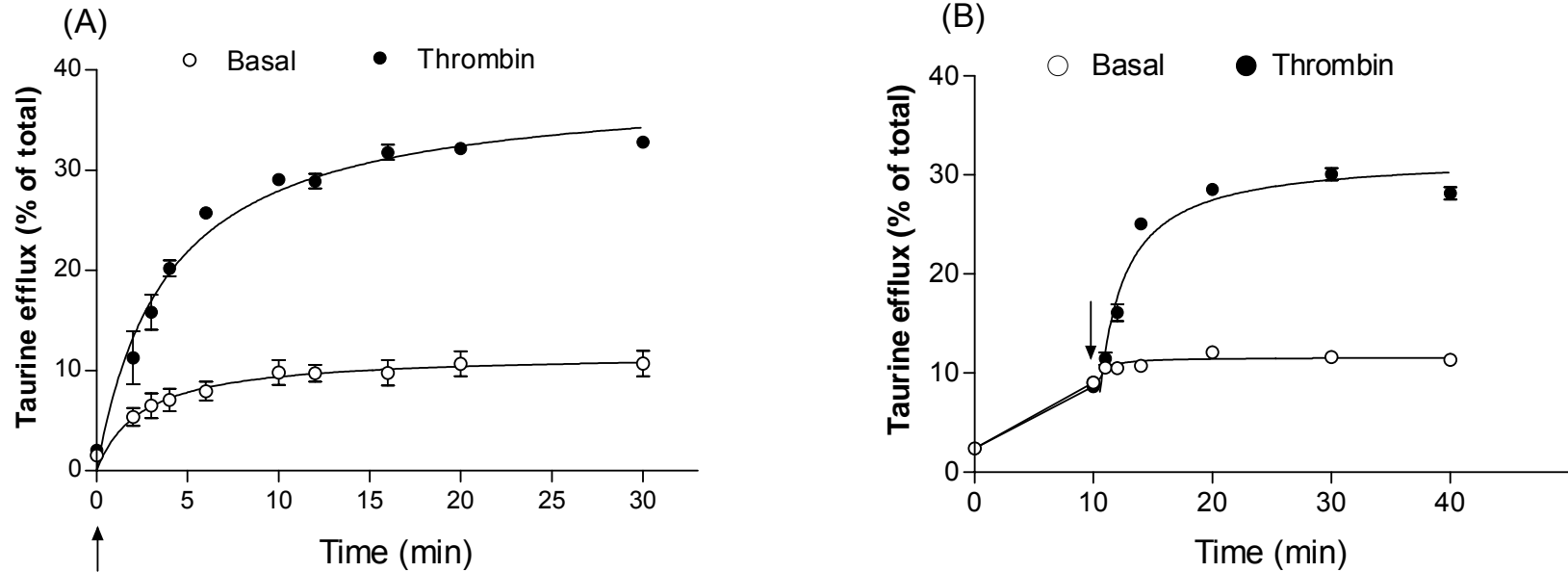
basal release of taurine is not dependent on either parameter. This suggests that the ability of thrombin to activate VSOAC involves signaling pathways that are distinct from those elicited by hypotonicity alone. A requirement for  $\text{Ca}^{2+}$  and PKC in receptor-regulated osmolyte release is emerging as a general characteristic (Loveday et al., 2003; Mongin and Kimelberg, 2005). However, the source of  $\text{Ca}^{2+}$  may differ depending upon the receptor and/or cell type (Loveday et al., 2003). Both PKC and  $\text{Ca}^{2+}$  CaMKII are potential downstream targets for  $\text{Ca}^{2+}$  activation. However, the inability of KN-93 to inhibit thrombin-stimulated taurine efflux suggests that  $\text{Ca}^{2+}$  CaMKII is not involved in VSOAC regulation.

Although the general characteristics of swelling-induced osmolyte release from neural cells have been extensively examined, only recently has evidence emerged that certain pharmacologically distinct receptors such as the P2Y purinergic,  $\text{M}_3$ -mAChR and now PAR-1, are able to positively regulate osmolyte efflux. There are two major implications of these findings. The first is that the ability of receptors to stimulate osmolyte release suggests that the process *in vivo* whereby hypoosmotically-stressed cells restore their volume may be more dynamic than previously considered, since these cells are also likely to be continuously subjected to neurohumoral regulation. The second is that the ability of these receptors to facilitate osmolyte release under isotonic conditions via VSOAC raises the possibility that receptor-mediated release of another quantitatively major osmolyte in the brain, namely glutamate, which also functions as a neurotransmitter, may constitute a means for intercellular signaling between glia and neurons. While this possibility has previously been raised for

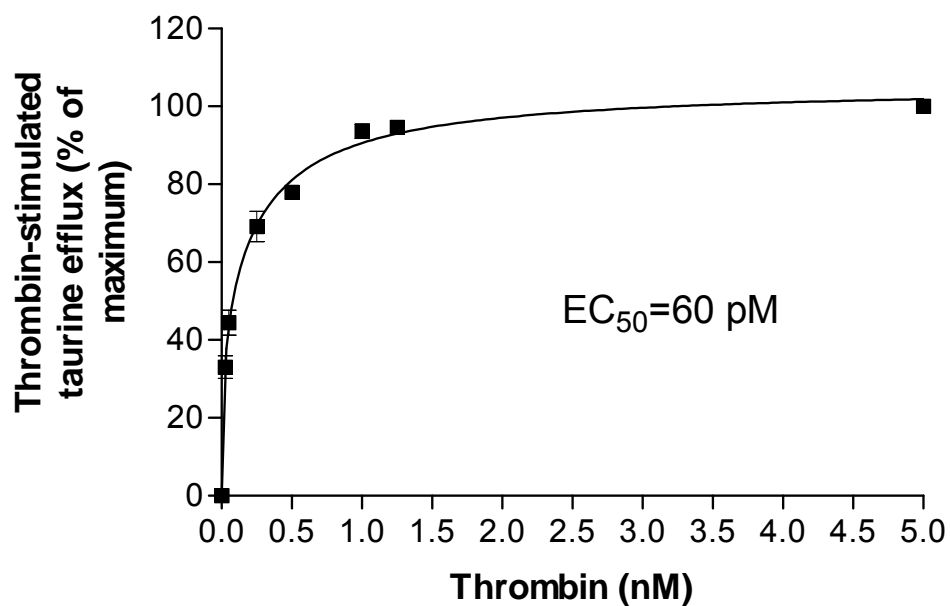


ATP modulation of glutamate release from astrocytes under isotonic conditions (Jeremic et al., 2001), the present data raises the possibility that additional receptors may also share this property. In addition, it should be noted that taurine itself has agonist properties at both GABA<sub>A</sub> and glycine receptors (Hussy et al., 1997; Hilgier et al., 2005).

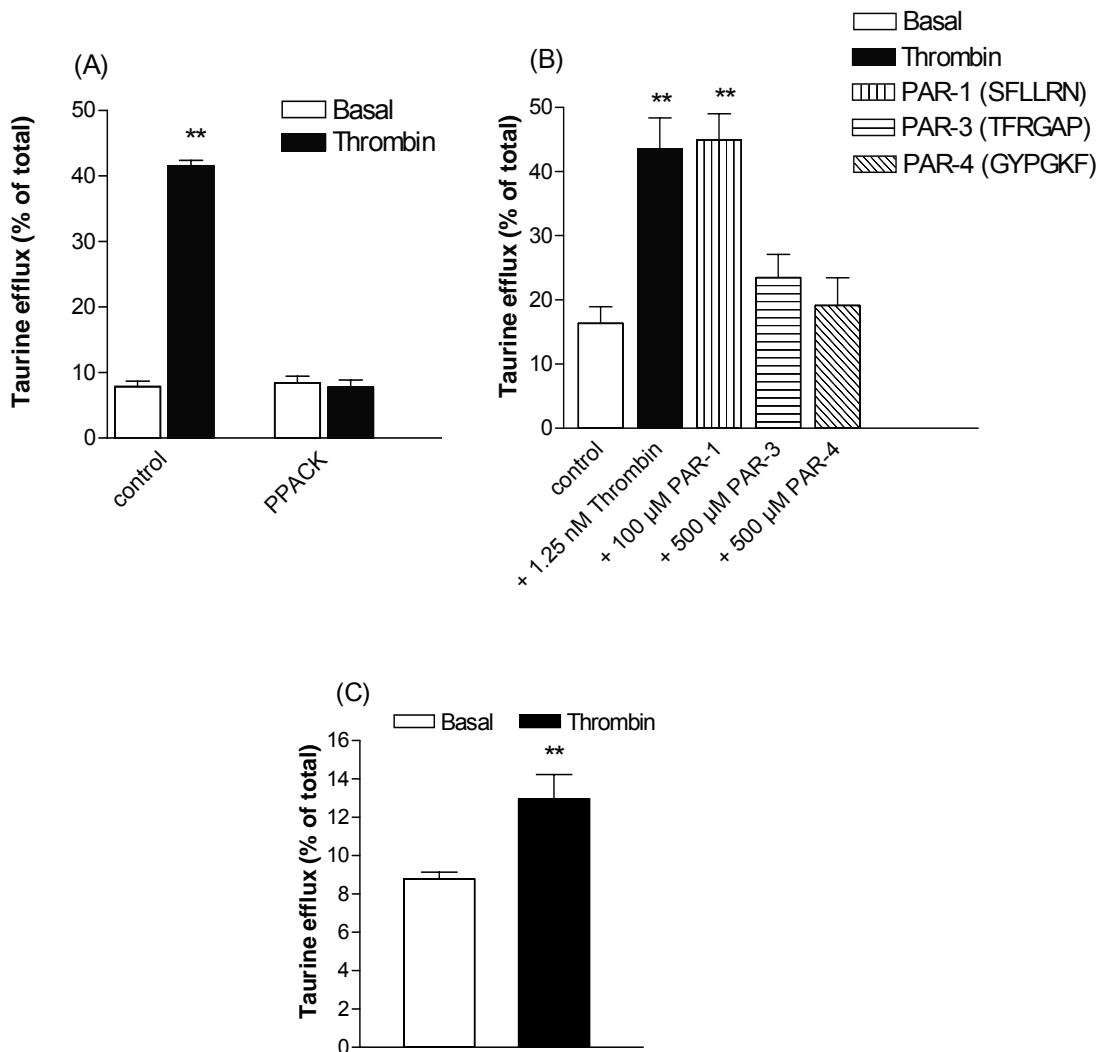
In summary, the present data emphasizes the importance of receptor-regulation of osmolyte release through a VSOAC. Control of neural cell swelling is of particular importance to the CNS due to the spatial restrictions of the skull. In this case, thrombin via its PAR-1 receptor plays a role in protection of neural cells from osmotic insults and regulates volume via a mechanism dependent upon intracellular Ca<sup>2+</sup> and PKC. This role for thrombin in osmoregulation within the brain adds to the growing list of functions attributed to this protease in CNS physiology and pathology.



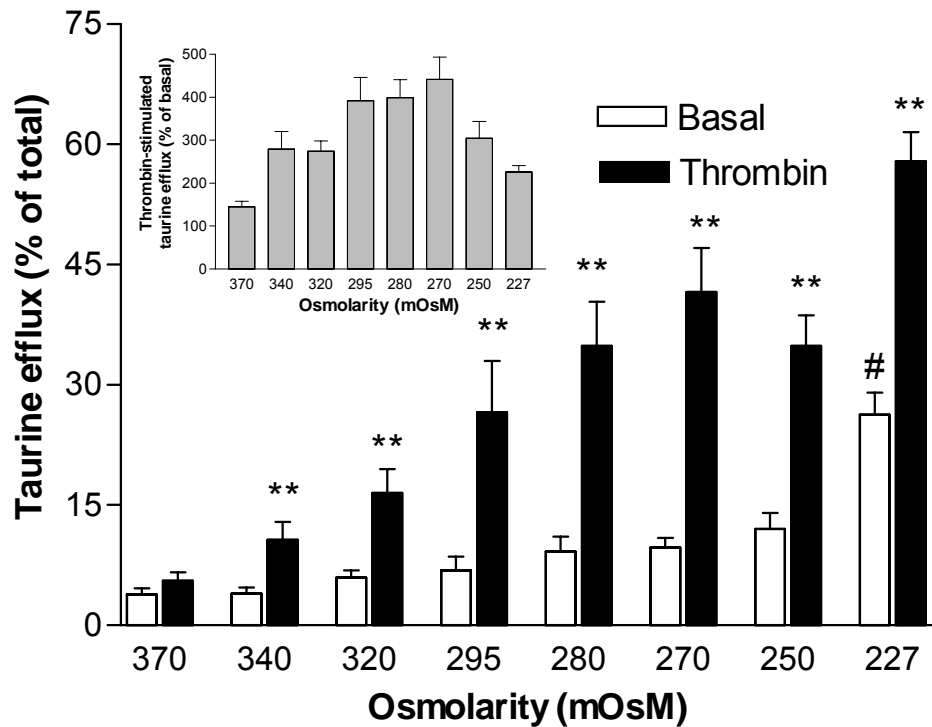
**Figure 2.1. Kinetics of basal- and thrombin-stimulated taurine efflux from human 1321N1 astrocytoma cells.** (A) 1321N1 human astrocytoma cells that had been prelabeled in the presence of [ $^3$ H]taurine were washed twice with 2 ml of isotonic buffer A before incubation in 270 mOsm buffer A in the presence or absence of 1.25 nM thrombin (added at time zero, as indicated by the arrow). Reactions were terminated at the times indicated and taurine efflux measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for triplicate replicates. Data shown are representative of three experiments. (B) Cells were treated as described in (A) with the exception that cells were allowed to incubate for 10 min in hypotonic buffer A (270 mOsm) prior to the addition of thrombin (indicated by arrow).



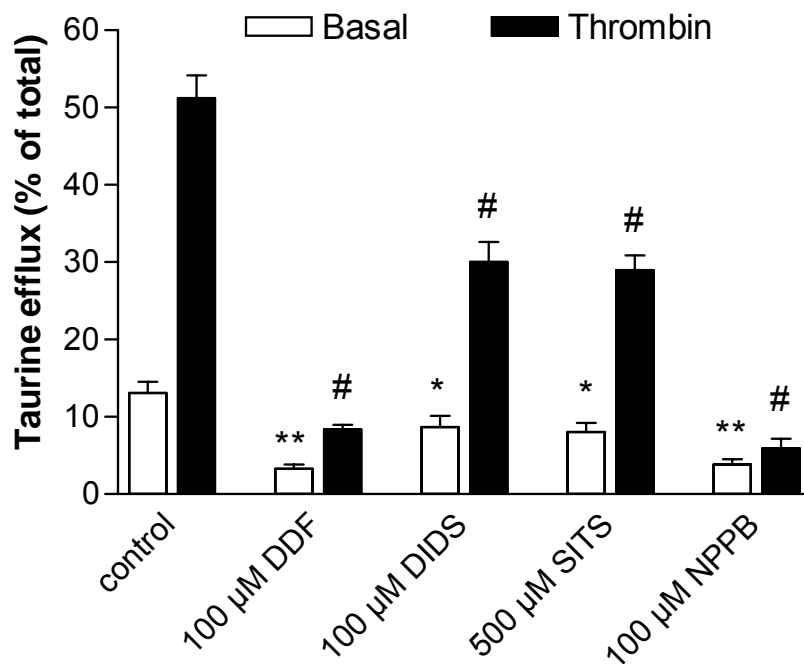
**Figure 2.2. Concentration-response relationship for thrombin-stimulated taurine efflux.** Cells that had been prelabeled with [<sup>3</sup>H]taurine were washed with isotonic buffer A and then incubated in 270 mOsM buffer in the presence of thrombin at the concentrations indicated. Reactions were terminated after 20 min and taurine efflux was monitored. Results are expressed as percentage of maximum agonist response (obtained at 5 nM thrombin) and are the means  $\pm$  S.E.M. for three independent experiments. The calculated EC<sub>50</sub> value for stimulated taurine efflux was 0.06 nM.



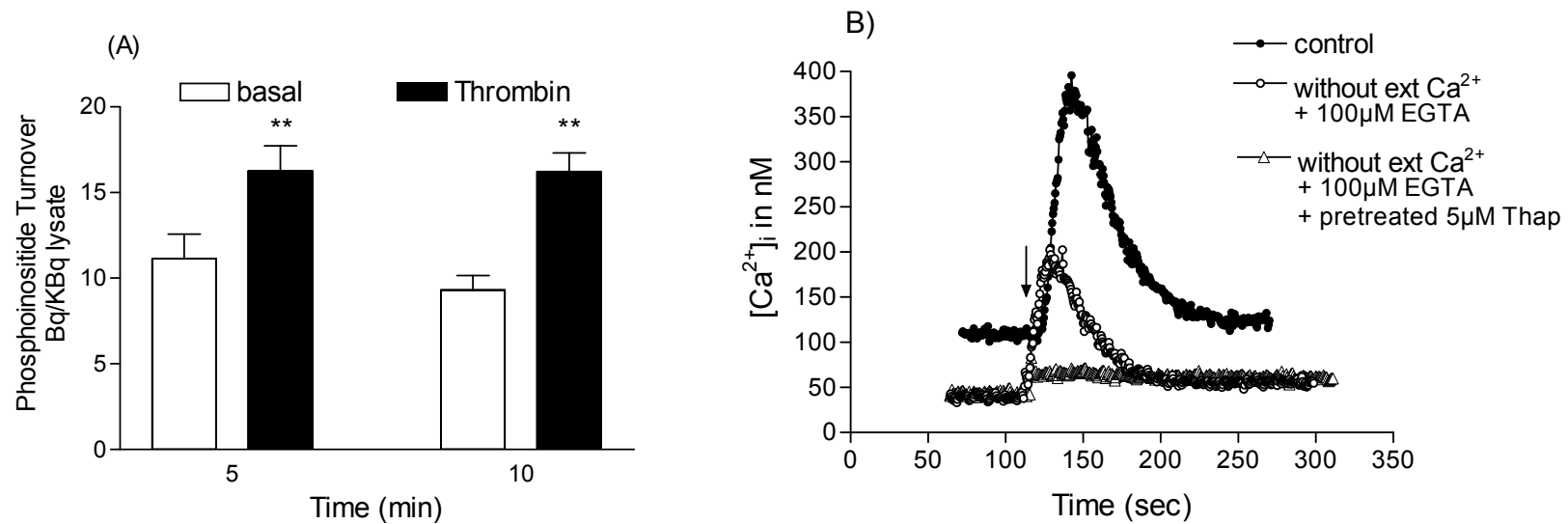
**Figure 2.3. Thrombin enhances taurine efflux from 1321N1 astrocytoma through its protease activity and via a Proteinase Activated Receptor-1 subtype.** (A) Cells that had been pre-labeled with [ $^3$ H]taurine were washed in isotonic buffer A and incubated for 20 min in 270 mOsM buffer A in the presence or absence of 1.25 nM thrombin. In some experiments thrombin was pretreated with 4  $\mu$ M PPACK, a protease inhibitor. In (B) cells were incubated for 20 min in the presence of thrombin or synthetic peptides specific for PAR-1, PAR-3 and PAR-4 subtypes and efflux monitored. Maximally effective concentrations of the synthetic peptides were used. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M for 4 to 6 independent experiments. \*\*, different from basal control,  $p < 0.01$  (by repeated measures ANOVA followed by Dunnett's multiple comparisons test). In (C) primary cultures of rat astrocytes were pre-labeled overnight with [ $^3$ H]taurine, washed in isotonic buffer A and then incubated for 20 min in 270 mOsM buffer A in the presence or absence of 1.25nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M for 7 independent experiments. \*\*, different from basal control,  $p < 0.01$  (by paired student's t-test).



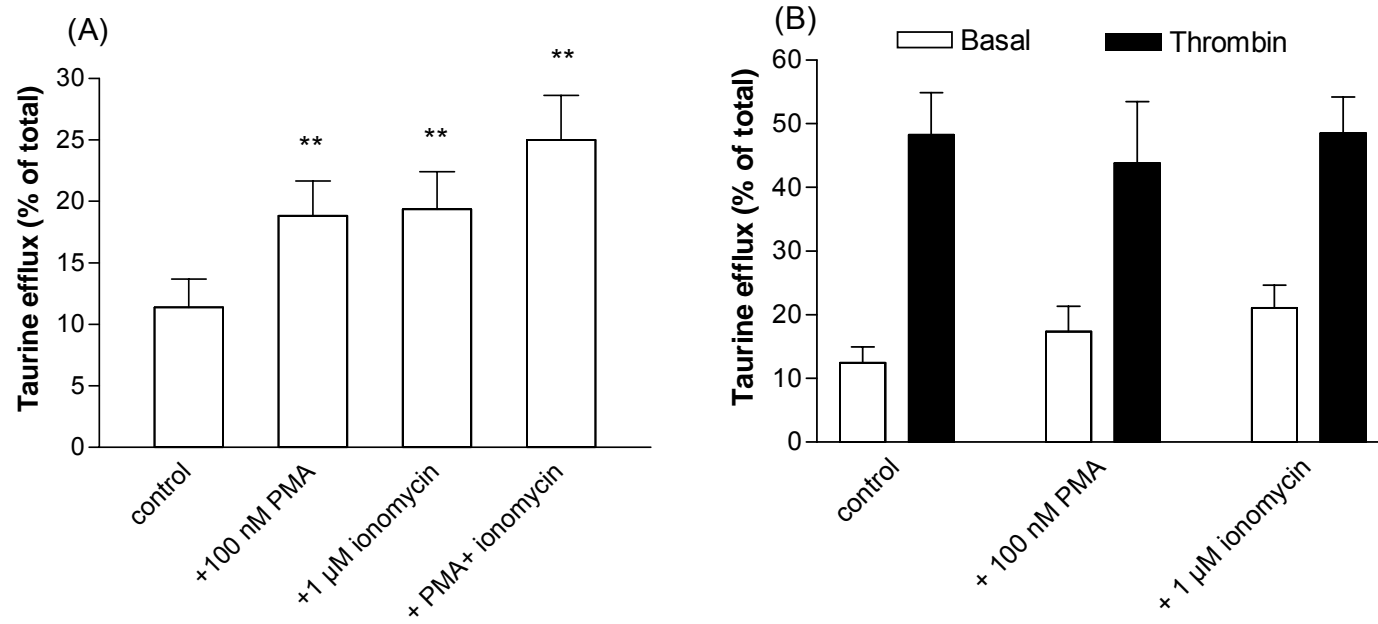
**Figure 2.4. Basal- and thrombin-stimulated release of taurine as a function of osmolarity.** Cells prelabeled with [<sup>3</sup>H]taurine were first washed in isotonic buffer A and then incubated for 20 min in buffer A at the osmolarities indicated in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 to 8 independent experiments, each performed in triplicate. #, different from taurine release observed in cells incubated in isotonic buffer A (340 mOsM),  $p < 0.01$  (by one-way ANOVA followed by Dunnett's multiple comparison test). \*\*, different from basal release (in the absence of thrombin-open bars),  $p < 0.01$  (by paired student's t-test). *Inset*-thrombin-stimulated efflux calculated as a percentage of basal, at each osmolarity.



**Figure 2.5. Inhibition of basal- and thrombin-stimulated taurine release by anion channel blockers.** Cells that had been prelabeled with [<sup>3</sup>H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (270 mOsM) with 100 μM dideoxyforskolin (DDF), 100 μM DIDS, 500 μM SITS or 100 μM NPPB in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 20 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of 6-8 independent experiments, each performed in triplicate. \* and \*\*, different from control basal, p<0.05 and p<0.01 respectively. #, different from thrombin-stimulated efflux under control conditions, p<0.01 (by one-way ANOVA followed by Dunnett's multiple comparisons test).

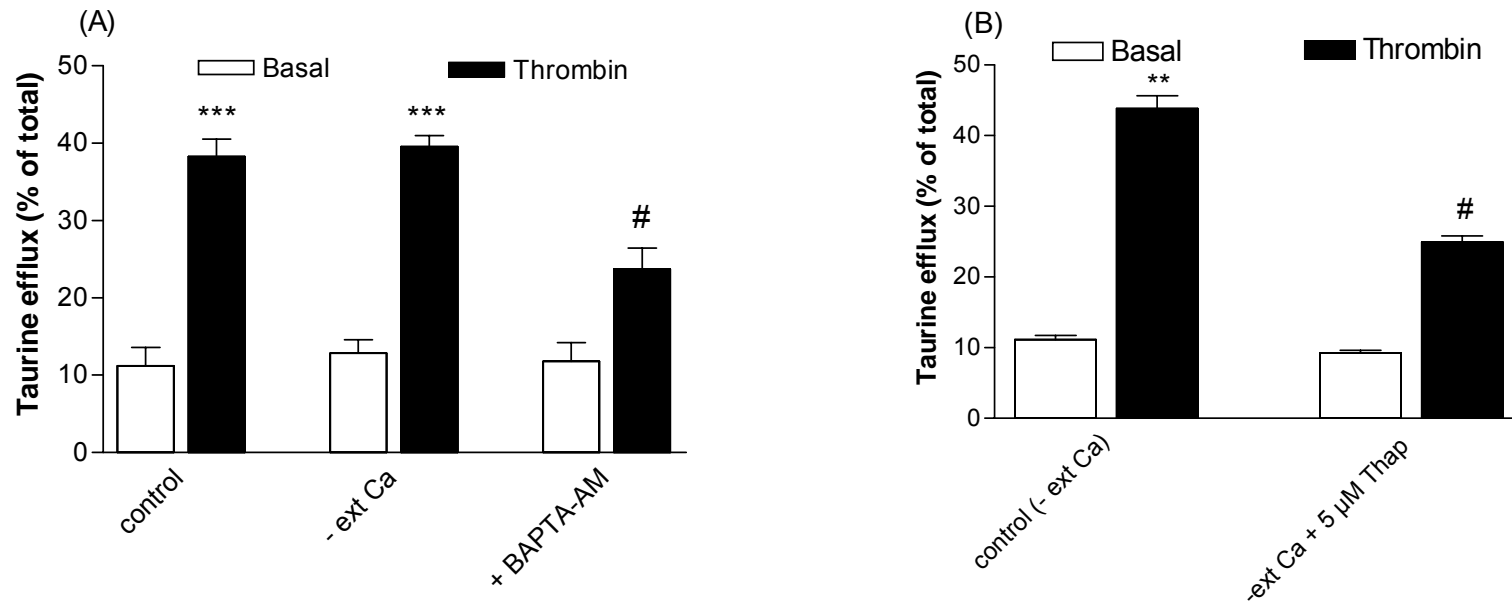


**Figure 2.6. Thrombin elicits an increase in phosphoinositide turnover and in cytoplasmic free calcium.** (A) Cells that had been prelabeled for 96 h with [<sup>3</sup>H]inositol were washed in isotonic Buffer A and then incubated for either 5 or 10 min in hypotonic buffer A (270 mOsM) in the presence or absence of thrombin (1.25 nM). Reactions were terminated by the addition of trichloroacetic acid and the accumulation of radiolabeled inositol phosphates was monitored as an index of stimulated phosphoinositide turnover. Results are expressed as inositol phosphate release/total soluble radioactivity and are the means  $\pm$  S.E.M. for five (5 min incubation) or four (10 minute incubation) independent experiments. \*\*, different from basal release,  $p < 0.001$  (by paired Student's *t* test). (B) Fura-2 loaded cells were resuspended in 270 mOsM buffer A with (●) or without (○) extracellular Ca<sup>2+</sup> or (△) pretreated 5 min with 5  $\mu$ M thapsigargin in the absence of extracellular Ca<sup>2+</sup>. Ca<sup>2+</sup> signals were monitored after the addition of thrombin (1.25 nM) at 120 sec (indicated by the arrow). Traces shown are representative of  $n = 8-14$  experiments obtained with 4-7 separate cell preparations.

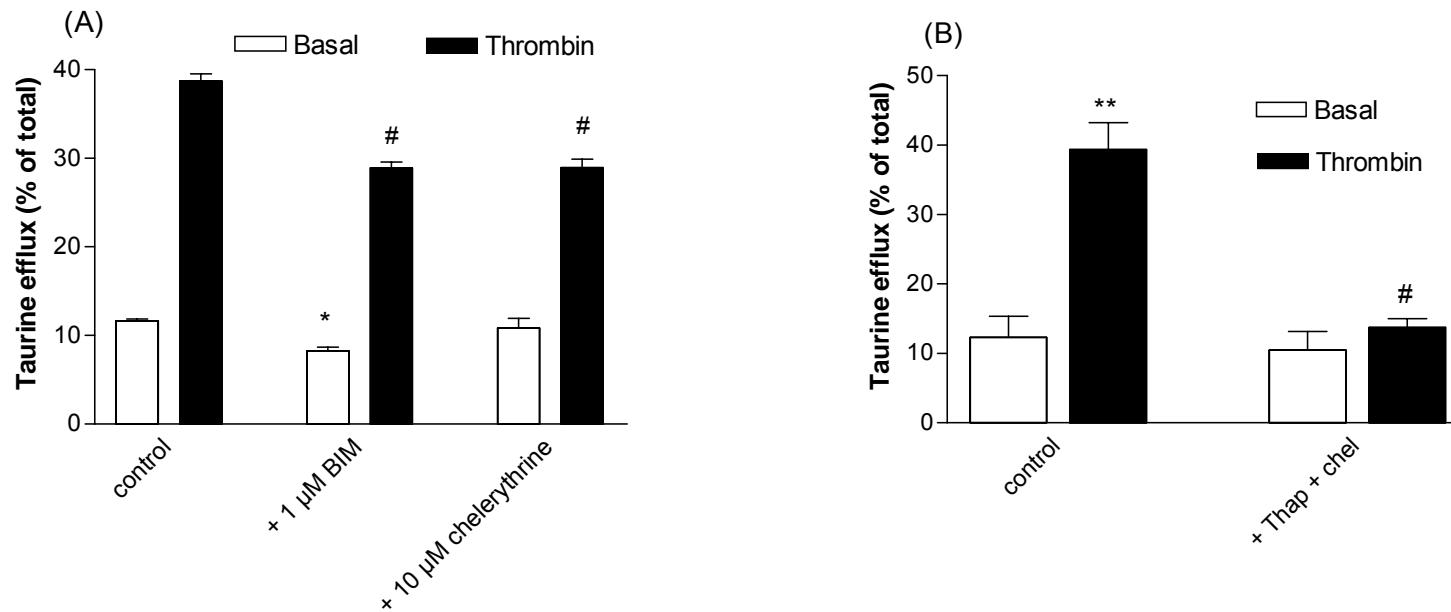


**Figure 2.7. Activation of either PKC, or a rise in intracellular  $\text{Ca}^{2+}$ , facilitates taurine efflux.** (A) Cells that had been prelabeled with [ $^3\text{H}$ ]taurine were washed with isotonic buffer A and then incubated in hypotonic buffer A (270 mOsM) with or without PMA (100 nM), ionomycin (1  $\mu\text{M}$ ) or both. Reactions were terminated after 20 min and taurine efflux monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 6 independent experiments, each performed in triplicate. \*\*, different from control,  $p < 0.01$  (by repeated measures ANOVA followed by Dunnett's multiple comparisons test). (B) Cells were treated as described in (A) but incubated with thrombin (1.25 nM), in the absence or presence of PMA or ionomycin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for four independent experiments. Thrombin-stimulated taurine efflux was not increased by the presence of either PMA or ionomycin.





**Figure 2.8. The role of extracellular- and intracellular calcium in thrombin-stimulated taurine efflux.** (A) Cells that had been prelabeled with [ $^3\text{H}$ ]taurine were washed in isotonic buffer A and then incubated for 20 min in hypotonic buffer A (270 mOsm) in the absence (-ext Ca:  $\text{Ca}^{2+}$  was omitted and 100  $\mu\text{M}$  EGTA added) or presence of extracellular  $\text{Ca}^{2+}$  and with the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. In some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 50  $\mu\text{M}$  BAPTA-AM prior to the measurement of efflux. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 3 to 6 independent experiments. Basal- (open bars) and thrombin-stimulated taurine release (filled bars) was monitored under all conditions. \*\*\*, different from control basal,  $p < 0.001$ , #, different from thrombin-stimulated efflux under control conditions,  $p < 0.05$  (by one-way ANOVA followed by Dunnett's multiple comparisons test). (B) Cells were preincubated for 5 min in 270 mOsm buffer A ( $\text{Ca}^{2+}$  omitted and 50  $\mu\text{M}$  EGTA added) in either the absence (control) or presence of 5  $\mu\text{M}$  thapsigargin. Thrombin or buffer A was then added and incubations allowed to proceed for an additional 10 min. Results are expressed as taurine efflux (percent of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 5 independent experiments. \*\*, different from control basal,  $p < 0.01$ ; #, different from thrombin-stimulated efflux under control conditions,  $p < 0.01$  (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).



**Figure 2.9. Inhibition of thrombin-stimulated taurine efflux by PKC inhibitors in the presence or absence of  $\text{Ca}^{2+}$ .** (A) Cells were pretreated with 10  $\mu\text{M}$  chelerythrine or 1  $\mu\text{M}$  BIM in isotonic buffer A for 15 min before incubation of cells in hypotonic buffer A (270 mOsm) in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 20 min and taurine efflux monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M for three independent experiments, each performed in triplicate. \*, different from basal control,  $p < 0.05$  and #, different from thrombin-stimulated efflux under control conditions,  $p < 0.01$  (by repeated measures ANOVA followed by Dunnett's multiple comparisons test). (B) Cells were first preincubated for 15 min in the absence (control) or presence of 10  $\mu\text{M}$  chelerythrine (chel) in isotonic buffer A. The medium was then aspirated and replaced with 270 mOsm buffer A, either  $\text{Ca}^{2+}$  containing (control) or with  $\text{Ca}^{2+}$  omitted and 50  $\mu\text{M}$  EGTA, 5  $\mu\text{M}$  thapsigargin and 10  $\mu\text{M}$  chelerythrine added. Cells were preincubated for 5 min prior to the addition of thrombin (final concentration 1.25 nM) or buffer A, prepared in the same media. After an additional 10 min, reactions were terminated and taurine efflux measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 6 independent experiments. \*, different from control basal release,  $p < 0.01$ ; #, different from thrombin-stimulated efflux under control conditions (repeated measures ANOVA followed by Dunnett's multiple comparisons test).

## References

- Cheema TA, Ward CA and Fisher SK (2005) Thrombin enhances taurine efflux through a Volume Sensitive Organic Anion Channel in human 1321N1 astrocytoma cells. 2005 Experimental Biology meeting abstracts. *The FASEB Journal*, Program # 628.9
- Coughlin SR (2000) Thrombin signaling and protease-activated receptors. *Nature* 407:258-264.
- Fisher SK, Domask LM and Roland RM (1989) Muscarinic receptor regulation of cytoplasmic  $Ca^{2+}$  concentrations in human SK-N-SH neuroblastoma cells:  $Ca^{2+}$  requirements for phospholipase C activation. *Mol Pharmacol* 35:195-204.
- Gingrich MB, and Traynelis SF (2000) Serine proteases and brain damage-is there a link? *Trends Neurosci* 23:399-407.
- Guo H, Liu D, Gelbard H, Cheng T, Insalaco R, Fernandez JA, Griffin JH and Zlokovic BV (2004) Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. *Neuron* 41:563-572.
- Heacock AM, Kerley D, Gurda GT, VanTroostenberghe AT, and Fisher SK (2004) Potentiation of the osmosensitive release of taurine and D-aspartate from SH-SY5Y neuroblastoma cells after activation of  $M_3$  muscarinic cholinergic receptors. *J Pharmacol Exp Ther* 311:1097-1104.
- Hilgier W, Oja SS, Saransaari P and Albrecht J (2005) Taurine prevents ammonia-induced accumulation of cyclic GMP in rat striatum by interaction with  $GABA_A$  and glycine receptors. *Brain Res* 1043: 242-6.
- Hollenberg MD and Compton SJ (2002) International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol Rev* 54: 203-217.
- Hussy N, Deleuze C, Pantaloni A, Desarmenien MG, Moos F (1997) Agonist action of taurine on glycine receptors in rat supraoptic magnocellular neurons: possible roles in osmoregulation. *J Physiol* 502: 609-21.
- Jeremic A, Jeftinija K, Stevanovic J, Glavaski A and Jeftinija S (2001) ATP stimulates calcium-dependent glutamate release from cultured astrocytes. *J Neurochem* 77:664-675.
- Jiang Y, Wu J, Hua Y, Keep RF, Xiang J, Hoff JT and Xi G (2002) Thrombin-receptor activation and thrombin-induced brain tolerance. *J Cereb Blood Flow Metab* 22:404-410.

- Jones LG, McDonough PM, and Brown JH (1989) Thrombin and trypsin act at the same site to stimulate phosphoinositide hydrolysis and calcium mobilization. *Mol Pharmacol* 36:142-149.
- Kimelberg HK (2000) Cell volume in the CNS: regulation and implications for nervous system function and pathology. *The Neuroscientist* 6:14-25.
- Lang, F., G. L. Busch, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D (1998) Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 78: 247-306.
- Lambert IH (2004) Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. *Neurochem Res* 29: 27-63.
- Loveday D, Heacock AM, and Fisher SK (2003) Activation of muscarinic cholinergic receptors enhances the volume-sensitive efflux of myo-inositol from SH-SY5Y neuroblastoma cells. *J Neurochem* 87:476-486.
- Macfarlane SR, Seatter MJ, Kanke T, Hunter GD and Plevin R (2001) Proteinase-activated receptors. *Pharmacol Rev* 53:245-282.
- Manolopoulos VG, Droogmans G and Nilius B (1997) Hypotonicity and thrombin activate taurine efflux in BC3H1 and C2C12 myoblasts that is down regulated during differentiation. *Biochem Biophys Res Commun* 232:74-79.
- Masada T, Xi G, Hua Y and Keep RF (2000) The effects of thrombin preconditioning on focal cerebral ischemia in rats. *Brain Res* 867:173-179.
- McManus ML, Churchwell KB and Strange K (1995) Regulation of cell volume in health and disease. *N Engl J Med* 333:1260-1266.
- Mongin AA and Kimelberg HK (2002) ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. *Am J Physiol Cell Physiol* 283:C569-C578.
- Mongin AA and Kimelberg HK (2005) ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple  $Ca^{2+}$ -sensitive mechanisms. *Am J Physiol Cell Physiol* 288:C204-C213.
- Moran J, Maar T and Pasantes-Morales H (1994) Cell volume regulation in taurine deficient cultured astrocytes. *Adv Exp Med Biol* 359: 361-7.
- Moran J, Maar TE and Pasantes-Morales H (1994) Impaired cell volume regulation in taurine deficient cultured astrocytes. *Neurochem Res* 19: 415-20.

- Nilius B and Droogmans G (2003) Amazing chloride channels: an overview. *Acta Physiol Scand* 177:119-147.
- Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V and Droogmans G (1997) Properties of volume-regulated anion channels in mammalian cells. *Prog Biophys Mol Biol* 68:69-119.
- Novak JE, Turner RS, Agranoff BW, and Fisher SK (1999) Differentiated human NT2-N neurons possess a high intracellular content of myo-inositol. *J Neurochem* 72: 1431-1440.
- Ossovskaya, V. S. and N. W. Bunnett (2004) Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* 84: 579-621.
- Pasantes-Morales H, Cardin V, and Tuz K (2000) Signaling events during swelling and regulatory volume decrease. *Neurochem Res* 25:1301-1314.
- Pasantes-Morales H, Franco R, Ordaz B, and Ochoa LD (2002) Mechanisms counteracting swelling in brain cells during hyponatremia. *Arch Med Res* 33:237-244.
- Striggow F, Riek M, Breder J, Henrich-Noack P, Reymann KG, and Reiser G (2000) The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations. *Proc Natl Acad Sci U S A* 97:2264-2269.
- Striggow F, Riek-Burchardt M, Kiesel A, Schmidt W, Henrich-Noack P, Breder J, Krug M, Reymann KG, and Reiser G (2001) Four different types of protease-activated receptors are widely expressed in the brain and are up-regulated in hippocampus by severe ischemia. *Eur J Neurosci* 14:595-608.
- Suo Z, Citron BA and Festoff BW (2004) "Thrombin: a potential proinflammatory mediator in neurotrauma and neurodegenerative disorders." *Curr Drug Targets Inflamm Allergy* 3:105-14.
- Thompson AK and Fisher SK (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositides turnover in two neuroblastomas: methodological considerations. *J Pharmacol Exp Ther* 252: 744-752.
- Trejo J (2003) Protease-activated receptors: New concepts in regulation of G protein-coupled receptor signaling and trafficking. *J Pharmacol Exp Ther* 307:437-442.

Vaughan PJ, Pike CJ, Cotman CW, and Cunningham DD (1995) Thrombin receptor activation protects neurons and astrocytes from cell death produced by environmental insults. *J Neurosci* 15:5389-5401.

Wang H and Reiser G (2003) Thrombin signaling in the brain: the role of protease-activated receptors. *Biol Chem* 384:193-202.

Xi G, Keep RF, Hua Y, Xiang J, and Hoff JT (1999) Attenuation of thrombin-induced brain edema by cerebral thrombin preconditioning. *Stroke* 30:1247-1255.

Xi G, Reiser G, and Keep RF (2003) The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J Neurochem* 84:3-9.

## CHAPTER 3

### RECEPTOR REGULATION OF THE VOLUME-SENSITIVE EFFLUX OF TAURINE AND IODIDE FROM HUMAN SH-SY5Y NEUROBLASTOMA CELLS: DIFFERENTIAL REQUIREMENTS FOR $\text{Ca}^{2+}$ AND PROTEIN KINASE C

#### Summary

The basal- (swelling-induced) and receptor-stimulated efflux of  $^{125}\text{I}^-$  and taurine have been monitored to determine whether these two osmolytes are released from human SH-SY5Y cells under hypotonic conditions via common or distinct mechanisms. Under basal conditions, both  $^{125}\text{I}^-$  (used as a tracer for  $\text{Cl}^-$ ) and taurine were released from the cells in a volume-dependent manner. Thrombin addition, mediated via the proteinase activated receptor-1 (PAR-1) subtype, significantly enhanced the release of both  $^{125}\text{I}^-$  and taurine (3- to 6-fold) and also increased the threshold osmolarity for efflux of these osmolytes ('set-point') from 200 to 290 mOsm. Inclusion of a variety of broad spectrum anion channel blockers and of 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB) attenuated the release of both  $^{125}\text{I}^-$  and taurine under basal- and receptor-stimulated conditions. Basal release of  $^{125}\text{I}^-$  and taurine was independent of  $\text{Ca}^{2+}$  or the activity of protein kinase C (PKC). However, although PAR-1-stimulated taurine efflux was attenuated by either a depletion of intracellular  $\text{Ca}^{2+}$  or inhibition of PKC by chelerythrine, the enhanced release of  $^{125}\text{I}^-$  was independent of both parameters. Stimulated efflux of  $^{125}\text{I}^-$

following activation of muscarinic cholinergic receptors was also markedly less dependent on  $\text{Ca}^{2+}$  availability and PKC activity than that observed for taurine release. These results indicate that, although the osmosensitive release of these two osmolytes from SH-SY5Y cells may occur via pharmacologically similar membrane channels, the receptor-mediated release of  $^{125}\text{I}^-$  and taurine is differentially regulated by PKC activity and  $\text{Ca}^{2+}$  availability.

### **Introduction**

Cell volume is constantly subject to change as a consequence of solute accumulation, oxidative metabolism or fluctuations in the osmolarity of the extracellular fluid. To survive, cells need to regulate their volume within relatively narrow limits and this homeostatic mechanism is of particular importance to the brain due to the restrictions of the skull. A common cause of brain swelling is hyponatremia, a condition that disproportionately impacts the elderly, infants, marathon runners and military personnel (Upadhyay et al., 2006). Hyponatremia is associated with a variety of neurological symptoms, such as disorientation, mental confusion and seizures (Kimelberg, 2000; Pasantes-Morales et al., 2000, 2002).

In response to hypotonic stress, cells swell with a magnitude proportional to the reduction in osmolarity. This is followed by a homeostatic mechanism termed regulatory volume decrease (RVD) which involves the extrusion of intracellular ions such as  $\text{K}^+$ ,  $\text{Cl}^-$  and a number of organic osmolytes, which together facilitate the loss of water to normalize cell volume (Pasantes-Morales et



al., 2000). Inorganic ions constitute two-thirds of the osmolytes released during RVD and the remainder is accounted for by “compatible” organic osmolytes such as polyols, methylamines and amino acids. Of these, taurine, an amino acid present in eukaryotic cells at concentrations of up to 40 mM, is considered to be an ideal osmolyte due to its metabolic inertness and abundance (Huxtable, 1992; Lambert, 2004).

It is proposed that extrusion of these osmolytes from the cell is mediated via a volume-sensitive organic osmolyte and anion channel (VSOAC), which is primarily permeable to  $\text{Cl}^-$  but impermeable to cations (for reviews, see Lang et al., 1998; Nilius and Droogmans, 2003). Evidence to support the involvement of VSOAC in response to hypotonic stress comes from studies in which RVD, volume-sensitive  $\text{Cl}^-$  current and organic osmolyte release can all be blocked by broad spectrum anion channel inhibitors, such as DDF or NPPB, and by a highly selective agent, DCPIB (Decher et al., 2001; Abdullaev et al., 2006). Similarities in the pharmacological inhibition profile of swelling-activated efflux of organic osmolytes and  $\text{Cl}^-$  in response to anion channel blockers has led to the suggestion that a common pathway exists for the extrusion of both  $\text{Cl}^-$  and organic osmolytes (Jackson and Strange, 1993; Banderali and Roy, 1992; Sanchez-Olea et al., 1996; Abdullaev et al., 2006). However this possibility is at variance with results obtained from some non-neural tissues in which  $\text{Cl}^-$  and taurine effluxes were found to exhibit differences in kinetics of release, osmotic sensitivity and/or degree of inhibition by anion channel blockers, results which suggest the existence of separate volume-sensitive channels for  $\text{Cl}^-$  and organic

osmolytes (Lambert and Hoffman, 1994; Davis-Amaral et al. 1996; Shennan et al., 1996; Stutzin et al., 1999; Shennan and Thomson, 2000; Tomassen et al., 2004).

When measured in vitro, the efflux of organic osmolytes is relatively insensitive to hypotonic stress often requiring substantial (>25%) reductions in osmolarity. However, recent studies from this and other laboratories have demonstrated that the volume-sensitive efflux of organic osmolytes from neural preparations can be enhanced following activation of cell-surface receptors. The latter include P2Y purinergic receptors in rat astrocytes (Mongin and Kimelberg, 2002, 2005), M<sub>3</sub> muscarinic cholinergic (mAChR), lysophosphatidic and sphingosine 1-phosphate receptors in human SH-SY5Y neuroblastoma cells (Loveday et al., 2003; Heacock et al., 2004, 2006) and proteinase-activated receptor-1 (PAR-1) in human 1321N1 astrocytoma and rat astrocytes (Cheema et al., 2005). In each case, Ca<sup>2+</sup> availability and PKC activity are required for the maximum release of organic osmolytes.

The goals of the present study were two-fold. First, to determine whether the release of <sup>125</sup>I<sup>-</sup> (used as a tracer for Cl<sup>-</sup>) from hypotonically-stressed SH-SY5Y neuroblastoma cells was, like that of taurine, subject to receptor regulation and second, to evaluate whether these two osmolytes are released from the cells via similar or distinct mechanisms. The results indicate that the activation of either PAR-1 or mAChRs elicits a significant increase in the osmosensitive release of both <sup>125</sup>I<sup>-</sup> and taurine and that the efflux of these osmolytes exhibits a similar, if not identical, inhibition profile in response to a variety of putative pharmacological

inhibitors of VSOAC. However, the receptor-mediated efflux of  $^{125}\text{I}^-$  can be readily differentiated from that of taurine on the basis of its more limited dependence on  $\text{Ca}^{2+}$  availability and, to a lesser extent, PKC activity. Thus, in SH-SY5Y cells, although both osmolytes may exit via a common (or pharmacologically similar) channel(s), distinct biochemical requirements exist for the receptor-stimulated release of  $^{125}\text{I}^-$  and taurine.

### Methods

**Materials.** [1,2- $^3\text{H}$ ] Taurine (1.15 TBq/ml) and Na iodide ( $^{125}\text{I}^-$  labeled; 3885 MBq/ml) were obtained from Amersham Biosciences (Piscataway, NJ). Chelerythrine, thapsigargin, toxin B, Y-27632 and niflumic acid were obtained from Calbiochem (San Diego, CA). Thrombin, DIDS, NPPB, 1,9-Dideoxyforskolin and oxotremorine-M (Oxo-M) were purchased from Sigma-Aldrich (St. Louis, MO). DCPIB was obtained from Tocris Biosciences (Ellisville, MO). Thrombin receptor activating peptides; TFLLRN, TFRGAP, GYPGKF were purchased from BaChem (Torrance, CA). Fura 2/acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (Franklin Lakes, NJ). Universol was obtained from Valeant Pharmaceuticals (Costa Mesa, CA).

**Cell culture conditions.**

Human SH-SY5Y neuroblastoma cells (passages 75-90) were grown in tissue culture flasks (75 cm<sup>2</sup>/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330- 340 mOsm. Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was aspirated and the cells detached from the flask with a tryPLE express (Biowhittaker, MD) or sterile D1 solution (Heacock et al., 2004). Cells were then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates for 5-6 days. Experiments were routinely conducted on cells that had reached 70–90% confluency.

**Measurement of efflux of taurine or <sup>125</sup>I<sup>-</sup>.**

Osmolyte efflux from SH-SY5Y neuroblastoma cells was monitored essentially as previously described (Heacock et al., 2004; Tomassen et al., 2004). In brief, cells were prelabeled overnight with 18.5 KBq/ml of [<sup>3</sup>H]taurine or 92.5 KBq/ml of <sup>125</sup>I<sup>-</sup> at 37°C. After prelabeling, the cells were washed 2 or 3 times with 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl<sub>2</sub>, 3.6 mM NaHCO<sub>3</sub>, 1mM MgCl<sub>2</sub> and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 340 mOsm). Cells were then allowed to incubate in 2 ml of hypotonic buffer A (295-195 mOsm; rendered hypotonic by a reduction in NaCl concentration) in the absence or presence of thrombin or Oxo-M. In some experiments, buffer A was

made hypertonic (370 mOsm) by the addition of NaCl. Osmolarities of buffer A were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots of the extracellular medium (200  $\mu$ l for taurine and 1 ml for  $^{125}\text{I}^-$ ) were removed and radioactivity determined after the addition of 6 ml Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 ml of ice-cold 6% (wt/vol) trichloroacetic acid for taurine or 1 ml of 0.1 M NaOH for  $^{125}\text{I}^-$ . Efflux of taurine or  $^{125}\text{I}^-$  was calculated as a fractional release, i.e., the radioactivity released in the extracellular medium as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay. For  $^{125}\text{I}^-$  efflux, radioactivity released at the zero time point was subtracted from the observed release of  $^{125}\text{I}^-$ . Throughout this study, “basal” release of taurine or  $^{125}\text{I}^-$  is defined as that which occurs at a specified osmolarity in the absence of agonists.

#### **Measurement of Phosphoinositide Turnover.**

To monitor phosphoinositide turnover, SH-SY5Y cells that had been prelabeled with 148 KBq/ml of [ $^3\text{H}$ ]inositol for 96 hr were incubated in hypotonic buffer A (230 mOsm) that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher, 1990).

### **Measurement of Cytoplasmic Calcium Concentration.**

Cytoplasmic free calcium concentrations,  $[Ca^{2+}]_i$ , were determined in suspensions of SH-SY5Y neuroblastoma cells after preloading cells with the  $Ca^{2+}$  indicator, fura-2 AM (Molecular Probes, OR), as previously described (Fisher et al., 1989; Cheema et al., 2005). The fluorometer used was a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD).

### **Data analysis.**

Experiments were performed in triplicate and repeated at least three times. Values quoted are given as means  $\pm$  S.E.M. for the number (n) of independent experiments indicated. A two-tailed Student's t-test (paired) was used to evaluate differences between two experimental groups (level of significance,  $p < 0.05$ ). One-way or repeated measures Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups (GraphPad InStat Software, Inc. San Diego, CA)

## **Results**

**Volume-sensitive efflux of taurine and  $^{125}I^-$  from SH-SY5Y neuroblastoma cells is enhanced by the addition of thrombin.** When SH-SY5Y cells that had been pre-labeled with  $[^3H]$ taurine were exposed to hypotonic buffer A (230 mOsm; ~30% reduction in osmolarity), there was a time-dependent release of the radiolabeled amino acid from the cells (Fig. 3.1A). Although the presence of a

functionally coupled thrombin receptor on SH-SY5Y cells has not previously been reported, inclusion of thrombin (0.25U/ml, equivalent to 1.25 nM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of efflux by approximately 7-8 fold over basal (basal release is that monitored in the absence of thrombin). Similarly, exposure of the cells to hypotonic buffer A alone also resulted in an increase in  $^{125}\text{I}^-$  efflux (Fig. 3.1A) and this was enhanced by the presence of thrombin (2-3 fold). Both the rate and magnitude of thrombin-stimulated  $^{125}\text{I}^-$  efflux was greater than that of taurine release. Thus, the *net* increase in  $^{125}\text{I}^-$  efflux over basal due to the addition of thrombin reached a maximum of 42% of the total radioactivity within 5 min, whereas the corresponding value for taurine was 25% (Fig. 3.1B). Since the greatest difference in magnitude of thrombin-stimulated  $^{125}\text{I}^-$  and taurine release was observed in the first 5 min, the efflux of these osmolytes was subsequently routinely monitored after 5 min of incubation.

**Thrombin enhances the volume-sensitive release of taurine and  $^{125}\text{I}^-$  via the PAR-1 receptor.** To determine whether the ability of thrombin to enhance taurine and  $^{125}\text{I}^-$  release was mediated via the same receptor, three specific Protease-Activated Receptor (PAR) peptides were used. Addition of 100  $\mu\text{M}$  TFFLRN, a synthetic peptide specific for the PAR-1 subtype, significantly increased the release of both taurine (1226% of basal; Fig 3.2A) and  $^{125}\text{I}^-$  (278% of basal; Fig. 3.2B). The enhancement of osmolyte release obtained with a PAR-1-specific peptide was similar in magnitude to that observed for thrombin.

Although inclusion of a 500  $\mu\text{M}$  concentration of the PAR-3 specific peptide (TFRGAP) also increased the release of taurine (269% of basal) and  $^{125}\text{I}^-$  (142% of basal), the effect was markedly less than that obtained for the PAR-1 agonist. The addition of the PAR-4 peptide, GYPGKF, did not significantly increase taurine or  $^{125}\text{I}^-$  release. Taken collectively, these data indicate that the ability of thrombin to enhance taurine and  $^{125}\text{I}^-$  release in SH-SY5Y neuroblastoma cells is mediated primarily by the PAR-1 receptor subtype.

**Comparison of the volume-sensitive efflux of taurine and  $^{125}\text{I}^-$  at various osmolarities.** Because the degree of receptor-mediated facilitation of osmolyte release appears to be dependent on the degree of hypoosmotic stress in SH-SY5Y cells (Heacock et al., 2004; 2006), the ability of thrombin to potentiate the release of taurine (Fig. 3.3A) and  $^{125}\text{I}^-$  (Fig. 3.3B) at different osmolarities was examined. Both basal- and thrombin-stimulated release of taurine and  $^{125}\text{I}^-$  were monitored under conditions of isotonicity (340 mOsM: defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild - severe hypotonicity (295-195 mOsM) or mild hypertonicity (370 mOsM). In the series of experiments conducted, the basal release of taurine was not appreciably enhanced until the osmolarity of the buffer had been reduced to 195 mOsM (Fig. 3.3A). In contrast, the addition of thrombin resulted in a significant increase in taurine efflux (312% of control) even under mild hypotonic conditions (295 mOsM). Moreover, as the osmolarity of the buffer was reduced, the ability of thrombin to enhance taurine efflux over the basal component was further



increased. A similar trend was observed for  $^{125}\text{I}^-$  efflux for which the basal release was not significantly enhanced until the osmolarity of the buffer had been reduced to 200 mOsM (Fig. 3.3B). The addition of thrombin resulted in a significant increase in  $^{125}\text{I}^-$  efflux (183% of control) under mild hypotonic conditions (290 mOsM). The maximum enhancement of both taurine efflux (892% of control) and  $^{125}\text{I}^-$  (319% of control) in the presence of thrombin was observed at an osmolarity of approximately 230 mOsM. In contrast, when cells were exposed to mildly hypertonic buffer A (370 mOsM), the addition of thrombin did not significantly enhance the release of either taurine or  $^{125}\text{I}^-$ .

**Volume-sensitive efflux of taurine and  $^{125}\text{I}^-$  efflux from SH-SY5Y neuroblastoma is mediated via a VSOAC.** Since VSOAC is considered to be primarily a chloride channel, the ability of a variety of broad spectrum chloride channel inhibitors to attenuate basal- and thrombin-stimulated taurine (Fig. 3.4A) and  $^{125}\text{I}^-$  release was examined (Fig. 3.4B). The addition of DIDS, NPPB or DDF resulted in a significant inhibition of the basal- and thrombin-stimulated release of both taurine and  $^{125}\text{I}^-$  from SH-SY5Y cells (28-73% and 28-95% for basal- and thrombin-stimulated efflux, respectively; Fig. 3.4 A,B). In general, the anion channel blockers, in particular DIDS, were less effective inhibitors of  $^{125}\text{I}^-$  release than that of taurine under both basal- and agonist-stimulated conditions. The inclusion of 100  $\mu\text{M}$  niflumic acid, which, at this concentration is purported to inhibit  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Large and Wang, 1996) resulted in a 43% inhibition of thrombin-stimulated taurine release, but had no effect on either the

thrombin-stimulated  $^{125}\text{I}^-$  efflux or on the basal release of either osmolyte (Fig. 3.4 A, B).

Because these anion channel inhibitors are relatively non-specific, the ability of DCPIB, an agent that is considered highly selective for VSOAC (Decher et al., 2001; Best et al., 2004; Abdullaev et al., 2006), was also examined for its ability to inhibit both taurine and  $^{125}\text{I}^-$  efflux. Inclusion of 20  $\mu\text{M}$  DCPIB significantly inhibited the basal release of both taurine and  $^{125}\text{I}^-$  to a similar extent (49% and 58% inhibition; Fig. 3.5 A,B, respectively). Similarly, DCPIB also inhibited the thrombin-stimulated taurine and  $^{125}\text{I}^-$  release (85-87% inhibition).

**Thrombin addition elicits an increase in the concentration of intracellular calcium in SH-SY5Y cells via a phospholipase C - independent mechanism.**

As previously observed for 1321N1 astrocytoma cells (Cheema et al., 2005), the addition of thrombin to fura-2-loaded SH-SY5Y cells resulted in a significant increase in  $[\text{Ca}^{2+}]_i$  (from a basal value of 100 nM to a peak value of 250 nM, n = 8). Removal of extracellular  $\text{Ca}^{2+}$  diminished the thrombin-mediated increase in  $[\text{Ca}^{2+}]_i$  from 150 to 75 nM, (n = 8) whereas depletion of intracellular  $\text{Ca}^{2+}$  with thapsigargin completely abolished the ability of thrombin to increase  $[\text{Ca}^{2+}]_i$ . The thrombin-mediated rise in  $[\text{Ca}^{2+}]_i$  occurred independently of phospholipase C activation since no increase in release of inositol phosphates was observed in the presence of thrombin ( $104 \pm 2\%$  of control, n=3). In contrast, the addition of a 100  $\mu\text{M}$  concentration of the muscarinic agonist, Oxo-M, which also elicits a

robust increase in  $[Ca^{2+}]_i$  in these cells (Heacock et al., 2006), resulted in a significant increase in inositol phosphate release ( $250 \pm 19\%$  of control,  $n = 3$ ).

**Thrombin-stimulated efflux of taurine, but not that of  $^{125}I^-$ , is dependent on  $Ca^{2+}$  availability and activation of PKC.** Activation of thrombin receptors on 1321N1 astrocytoma cells has been reported to elicit an increase in taurine release that is dependent on the intracellular concentration of calcium and activation of PKC (Cheema et al., 2005). In agreement with our previous observations, the magnitude of thrombin-stimulated taurine release from SH-SY5Y neuroblastoma cells is also dependent on  $Ca^{2+}$  availability. However in SH-SY5Y cells, removal of extracellular  $Ca^{2+}$  alone is sufficient to inhibit thrombin-stimulated taurine release (24% inhibition), while the basal release of taurine is unaffected. Depletion of intracellular  $Ca^{2+}$  stores with 1  $\mu$ M thapsigargin did not further increase the extent of inhibition and no effect on basal release of taurine was observed (Fig. 3.6A). Neither the basal- nor thrombin-stimulated efflux of  $^{125}I^-$  efflux was attenuated by omission of extracellular  $Ca^{2+}$  or depletion of intracellular  $Ca^{2+}$  stores with 1  $\mu$ M thapsigargin (Fig. 3.6B).

To evaluate a role, if any, for PKC in basal and thrombin-stimulated efflux of taurine and  $^{125}I^-$ , cells were preincubated with 10  $\mu$ M chelerythrine prior to thrombin challenge under hypotonic conditions. Although chelerythrine had no effect on the basal release of taurine, it significantly attenuated thrombin-stimulated release (30% inhibition; Fig. 3.7A). In contrast, although a small

reduction (9%) in the basal release of  $^{125}\text{I}^-$  was observed following chelerythrine pretreatment, no effect on the magnitude of thrombin-stimulated efflux was observed (Fig. 3.7B). The combination of inhibition of PKC with 10  $\mu\text{M}$  chelerythrine, along with depletion of intracellular  $\text{Ca}^{2+}$  with 1  $\mu\text{M}$  thapsigargin, resulted in a 54% inhibition of thrombin-stimulated taurine release but had no effect on basal efflux (Fig. 3.8A). In contrast,  $^{125}\text{I}^-$  release elicited by the addition of thrombin was not attenuated under these conditions (Fig. 3.8B).

**Efflux of taurine and  $^{125}\text{I}^-$  following the activation of mAChRs is also differentially sensitive to depletion of  $\text{Ca}^{2+}$  and activation of PKC.** The observation that the efflux of taurine and  $^{125}\text{I}^-$  observed following thrombin addition is differentially regulated by  $\text{Ca}^{2+}$  and PKC prompted us to examine whether this relationship is also observed following the activation of mAChRs. As previously observed (Heacock et al., 2006), Oxo-M-stimulated taurine release was attenuated by omission of extracellular  $\text{Ca}^{2+}$  (60% inhibition; Fig. 3.9A) and further in the presence of 1  $\mu\text{M}$  thapsigargin to deplete intracellular  $\text{Ca}^{2+}$  pools ( $81 \pm 4\%$  inhibition; Fig. 3.9A). However, Oxo-M-stimulated  $^{125}\text{I}^-$  efflux was unaffected by removal of extracellular  $\text{Ca}^{2+}$  and significantly less inhibited than taurine release following the additional depletion of intracellular  $\text{Ca}^{2+}$  ( $31 \pm 6\%$  inhibition; Fig. 3.9B,  $p < 0.005$ ).

To examine the involvement of PKC, Oxo-M-stimulated taurine release was measured after preincubation of the cells with 10  $\mu\text{M}$  chelerythrine. Basal taurine release was unaffected whereas that due to Oxo-M addition was

significantly inhibited ( $73 \pm 5\%$  inhibition; Fig. 3.10A). Chelerythrine also significantly inhibited Oxo-M-stimulated  $^{125}\text{I}^-$  release ( $47 \pm 5\%$  inhibition; Fig. 10B) but the degree of inhibition was significantly less than that observed for taurine release ( $p < 0.05$ ). The combination of inhibition of PKC with  $10 \mu\text{M}$  chelerythrine, along with depletion of intracellular  $\text{Ca}^{2+}$  with  $1 \mu\text{M}$  thapsigargin, resulted in a  $94 \pm 3\%$  inhibition of Oxo-M-stimulated taurine release whereas stimulated  $^{125}\text{I}^-$  release was inhibited by  $64 \pm 1\%$  (Fig. 3.11 A,B,  $p < 0.001$  vs stimulated taurine release). Thus, a significant fraction (35-40%) of Oxo-M-stimulated  $^{125}\text{I}^-$  efflux, and all of that due to thrombin addition, is independent of both  $\text{Ca}^{2+}$  availability and PKC activity.

### Discussion

Previous studies of receptor-regulated osmolyte release from hypotonically-stressed neural cells have focused on the efflux of organic osmolytes, rather than that of  $\text{Cl}^-$ . In the present study, we demonstrate that the addition of thrombin to SH-SY5Y neuroblastoma cells results in a significant enhancement of the volume-sensitive efflux of both  $^{125}\text{I}^-$  and taurine and that for each osmolyte, stimulated release is mediated primarily by the PAR-1 subtype. However, receptor-mediated  $^{125}\text{I}^-$  efflux occurs more rapidly and to a greater extent than that of taurine (Fig. 3.1B), an observation that may reflect differences in the respective molecular sizes of the two osmolytes. A similar preferential release of  $^{125}\text{I}^-$  over that of taurine has previously been noted for HeLa and Intestinal 407 cells following cell swelling (Stutzin et al., 1999; Tomassen et al.,

2004). The threshold osmolarity ('set-point') at which the basal release of osmolytes occurs was the same for both  $^{125}\text{I}^-$  and taurine, i.e. approximately 200 mOsM (Fig. 3.4). This result is consistent with our previous studies with neurotumor cells in which a reduction in osmolarity of >25% was required to elicit a significant increase in osmolyte release (Heacock et al., 2004, 2006; Cheema et al., 2005). In contrast, in Intestinal 407 cells, the threshold osmolarity for release of  $^{125}\text{I}^-$  (260 mOsM) is reported to be higher than that of taurine (225 mOsM; Tomassen et al., 2004). In the present study, thrombin addition to SH-SY5Y cells increased the 'set point' for the efflux of both  $^{125}\text{I}^-$  and taurine from 200 mOsM to 290 mOsM. Thus receptor activation facilitates the release of both inorganic and organic osmolytes and this may constitute a mechanism whereby cells can respond to small changes in external osmolarity.

The possibility that the volume-sensitive release of  $\text{Cl}^-$  and organic osmolytes occurs via a common membrane channel (VSOAC) has received support primarily on the basis of the similarities of pharmacological inhibition profiles obtained in the presence of a variety of non-selective anion channel blockers (Banderali and Roy, 1992; Jackson and Strange, 1993; Sanchez-Olea et al., 1996; Abdullaev et al., 2006). However, in some tissues, the existence of separate  $\text{Cl}^-$  and taurine efflux pathways has also been proposed (Lambert and Hoffman, 1994; Stutzin et al., 1999; Shennan and Thomson, 2000; Tomassen et al., 2004). In addition, the issue of whether  $\text{Cl}^-$  and organic osmolytes are released from the cell under conditions of receptor activation via shared or distinct pathways has not yet been systematically addressed. In the present

study we observed that the inclusion of three anion channel blockers, namely DIDS, NPPB and DDF, inhibited both basal- and receptor-stimulated release of  $^{125}\text{I}^-$  and taurine. Of these, DDF and NPPB were more effective inhibitors than DIDS, particularly for stimulated  $^{125}\text{I}^-$  release. The sole agent that was able to differentiate between taurine and  $^{125}\text{I}^-$  release was niflumic acid, which, at the concentration employed (100  $\mu\text{M}$ ), is purported to inhibit  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Large and Wang, 1996). Although niflumic acid had no effect on the basal release of either osmolyte, it significantly inhibited thrombin-stimulated release of taurine, but not that of  $^{125}\text{I}^-$  (Fig. 3.5). However, the significance of this observation remains unclear for two reasons. First, DCPIB, a highly specific inhibitor of VSOAC that is without effect on  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels and other cation and anion channels (Decher et al., 2001; Best et al., 2004), was an equally effective inhibitor of basal-and receptor-stimulated release of both taurine and  $^{125}\text{I}^-$  from SH-SY5Y cells (Fig. 3.6). Second, niflumic acid attenuated thrombin-stimulated taurine efflux even under  $\text{Ca}^{2+}$ -depleted conditions, a result inconsistent with inhibition of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel (data not shown). Taken collectively, the most parsimonious interpretation of the current data is that, following receptor activation, both  $^{125}\text{I}^-$  and taurine are released from SH-SY5Y cells via the same (or pharmacologically indistinguishable) VSOAC channels.

Although the release of  $^{125}\text{I}^-$  and taurine exhibited a similar pharmacological inhibition profile, the receptor-mediated release of these two osmolytes could be readily differentiated on the basis of their dependence on

Ca<sup>2+</sup> availability and PKC activity. Previously, we and others have demonstrated that increases in [Ca<sup>2+</sup>]<sub>i</sub> or in PKC activity are not prerequisites for the basal (swelling-induced) release of organic osmolytes such as taurine and D-aspartate from neurotumor cells, neurons or astrocytes (Moran et al., 1997; Mongin and Kimelberg, 2002; Cardin et al., 2003; Loveday et al., 2003; Cheema et al., 2005). Similarly, in the present study, we observed that, at least under mildly hypotonic conditions, the basal release of <sup>125</sup>I<sup>-</sup> also appears to be essentially independent of Ca<sup>2+</sup> availability and PKC activity. However, PAR-1-mediated increases in taurine and <sup>125</sup>I<sup>-</sup> efflux differed in their dependence upon Ca<sup>2+</sup> availability and PKC activity. Thus, whereas taurine efflux was attenuated following the removal of extra- and intracellular Ca<sup>2+</sup>, or following inhibition of PKC activity with chelerythrine, thrombin-stimulated <sup>125</sup>I<sup>-</sup> efflux was unaffected by either treatment. Under conditions in which both Ca<sup>2+</sup> depletion and inhibition of PKC activity occurred, stimulated taurine efflux was inhibited by >50% whereas <sup>125</sup>I<sup>-</sup> release remained unchanged. Fura-2 fluorimetric studies indicated that the addition of thrombin to SH-SY5Y cells resulted in a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> (100 nM to 250 nM), which was abolished when both extra- and intracellular sources of Ca<sup>2+</sup> were depleted. Because the PAR-1-mediated increase in the release of <sup>125</sup>I<sup>-</sup> was not attenuated under these conditions, we conclude that the efflux of <sup>125</sup>I<sup>-</sup> (but not that of taurine) occurs independently of a rise in [Ca<sup>2+</sup>]<sub>i</sub> within these cells. This conclusion is consistent with the Ca<sup>2+</sup> insensitivity of thrombin-stimulated Cl<sup>-</sup> currents previously observed in pulmonary artery endothelial cells (Manolopoulos et al., 1997). Further evidence that Ca<sup>2+</sup> and

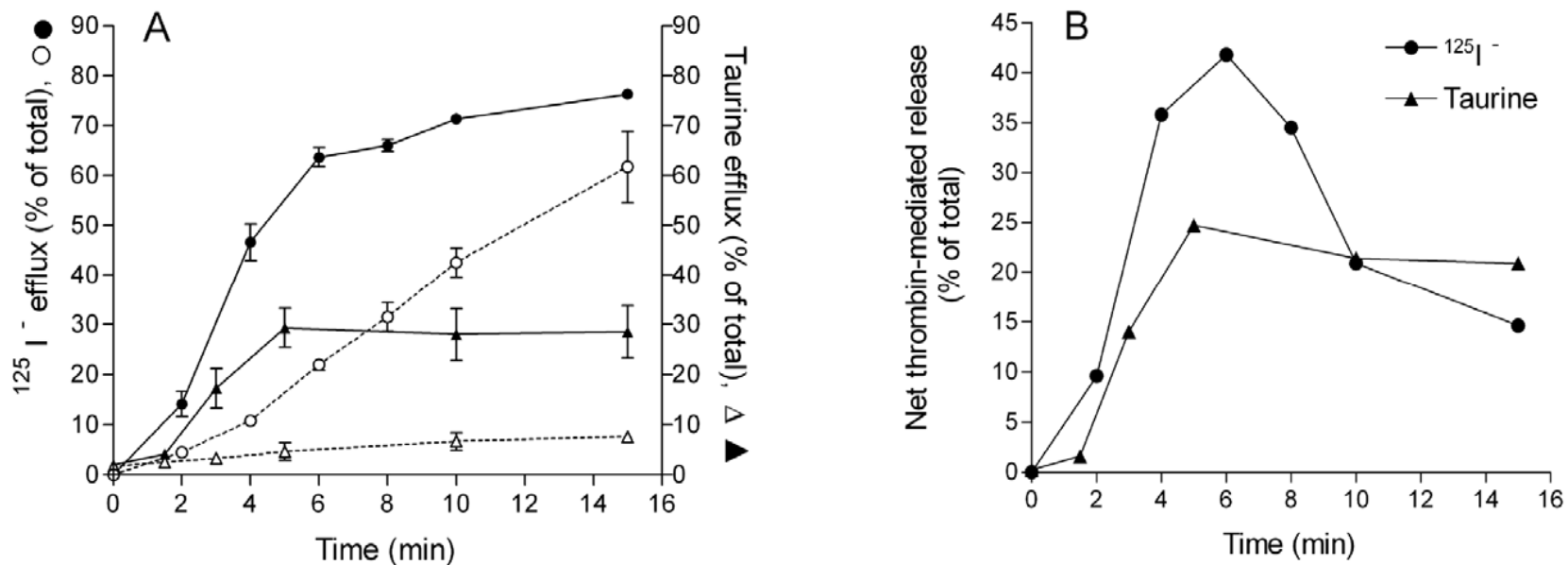


PKC differentially modulate the release of these two osmolytes from SH-SY5Y cells was obtained following the addition of the muscarinic agonist, Oxo-M. Activation of mAChRs on SH-SY5Y cells elicits a large increase in  $[Ca^{2+}]_i$  (from 100 to 450 nM), which is sustained due to a continuous influx of extracellular  $Ca^{2+}$  (Lambert and Nahorski, 1990; Heacock et al., 2006). Although omission of extracellular  $Ca^{2+}$  and depletion of intracellular  $Ca^{2+}$  with thapsigargin resulted in a pronounced inhibition of mAChR-stimulated taurine release (60 and 81% respectively), Oxo-M-stimulated  $^{125}I^-$  efflux was unaffected by removal of extracellular  $Ca^{2+}$  and much less inhibited (31%) by depletion of intracellular  $Ca^{2+}$  stores (Fig. 3.9). Similarly, inhibition of PKC resulted in a significantly greater loss of mAChR-stimulated taurine release (73%) than that of  $^{125}I^-$  efflux (47%).

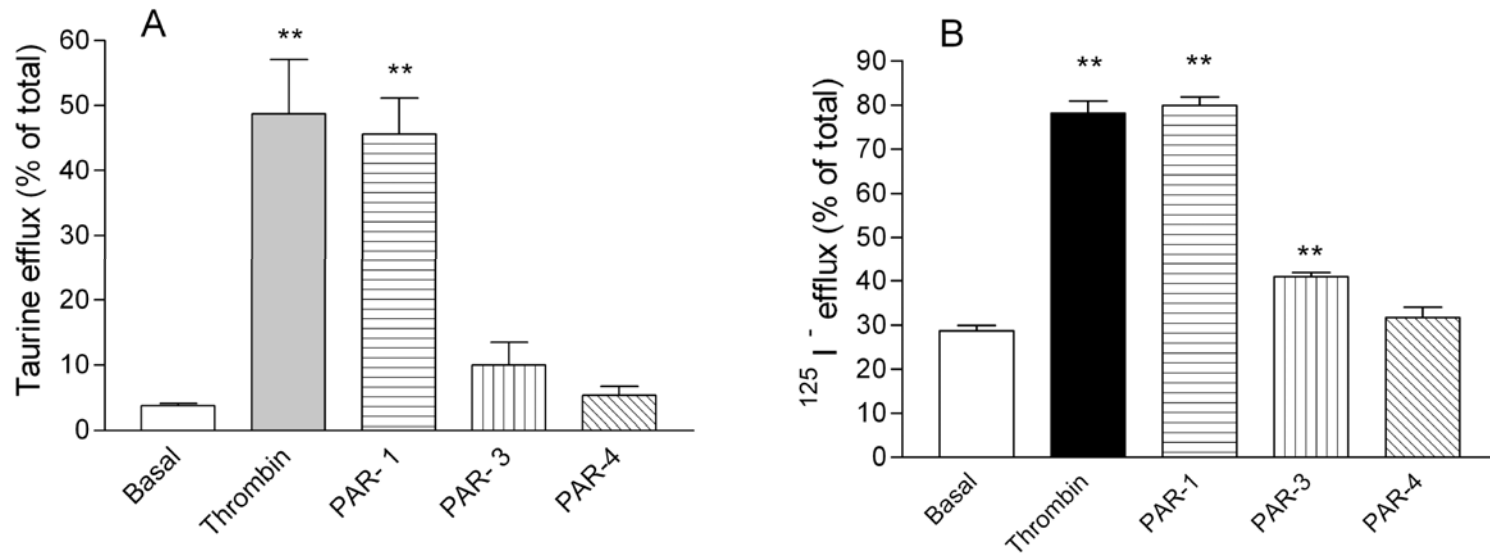
Two conclusions can be drawn from these results. The first is that, regardless of the receptor activated, the stimulated release of  $^{125}I^-$  is less dependent than taurine efflux on either  $Ca^{2+}$  availability or PKC activity. For the PAR-1 receptor, stimulated  $^{125}I^-$  efflux is fully independent of  $Ca^{2+}$  availability and PKC activity whereas for the mAChR, some degree of dependence upon these parameters is observed. The second conclusion is that although  $Ca^{2+}$  and PKC are required for maximum receptor activation of taurine efflux from SH-SY5Y cells, the degree of dependence is receptor-specific. Thus  $Ca^{2+}$  and PKC appear to play a quantitatively more significant role in mAChR stimulation of taurine release than that following the activation of either the PAR-1 or lysophospholipid receptors (Heacock et al., 2006).

Our observation that  $\text{Ca}^{2+}$  availability (and PKC activity) differentially regulate the receptor-stimulated release of taurine and  $^{125}\text{I}^-$  from SH-SY5Y cells is consistent with results previously obtained for hepatoma cells (Junankar et al., 2002). Osmotic swelling of these cells results in the release of intrinsic ATP, which subsequently activates P2Y receptors coupled to an increase in  $[\text{Ca}^{2+}]_i$ . However, although this rise in  $[\text{Ca}^{2+}]_i$  is required for the release of taurine, a stimulated efflux of  $^{125}\text{I}^-$  can occur in the absence of an increased intracellular  $\text{Ca}^{2+}$ . Conceivably, differences in  $\text{Ca}^{2+}$  and PKC requirements for taurine and  $^{125}\text{I}^-$  efflux in hepatoma and SH-SY5Y cells might reflect the following: (1) the receptor-specific activation of distinct signal transduction pathways ( $\text{Ca}^{2+}$ /PKC-dependent or –independent) that differentially contribute to the efflux of taurine and  $^{125}\text{I}^-$ , both of which are released through a common membrane channel, (2) the presence of separate, but pharmacologically similar, efflux channels for  $^{125}\text{I}^-$  and taurine that differ in their degree of regulation by  $\text{Ca}^{2+}$  and PKC or (3) a combination of both mechanisms (Fig. 3.12). In the context of multiple signaling pathways, one potential candidate, triggered by thrombin receptors, is rho-mediated remodeling of the cytoskeleton (Carton et al., 2002; Pederson et al., 2002). However, preincubation of SH-SY5Y cells with toxin B, or the rho kinase inhibitor Y-27632, had no effect on receptor-stimulated release of taurine or  $^{125}\text{I}^-$  (data not shown). The possibility that separate efflux channels mediate the release of taurine and  $^{125}\text{I}^-$  in SH-SY5Y cells has previously been suggested for non-neural cells (Lambert and Hoffman, 1994; Stutzin et al., 1999). Regardless of the pathways involved, our results indicate that, following receptor activation,

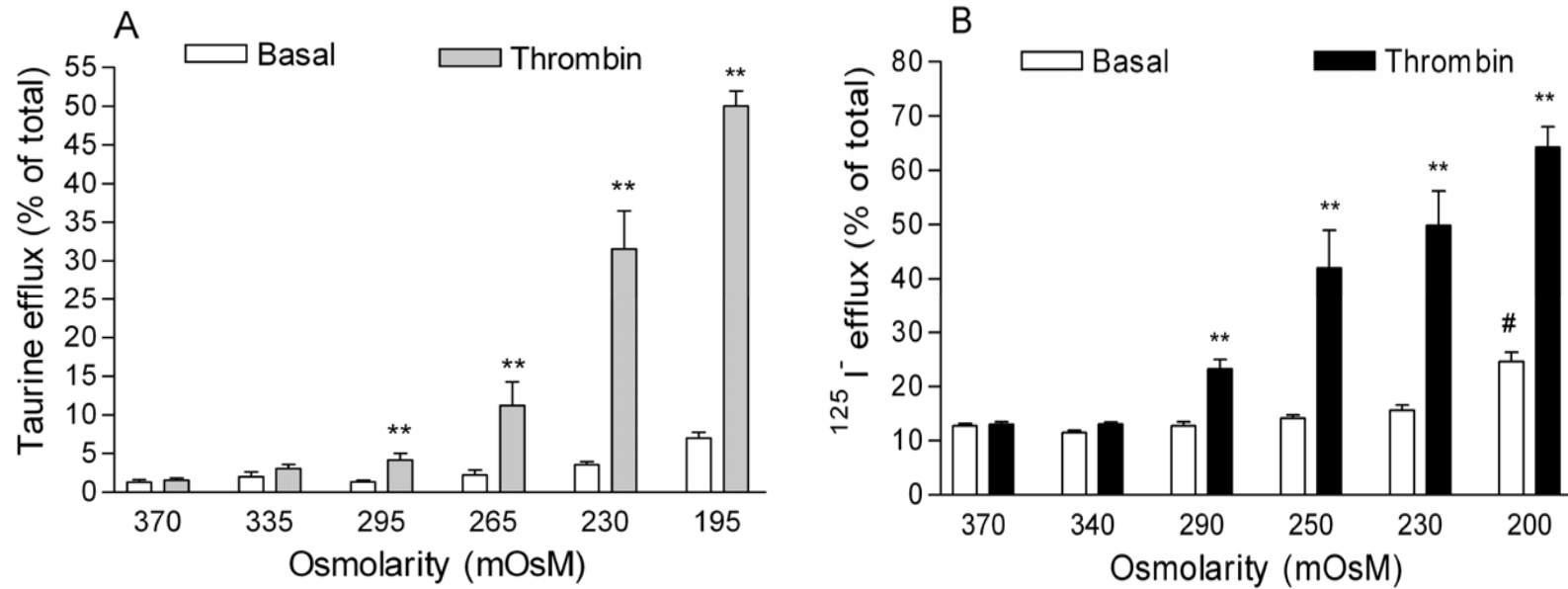
the volume-dependent release of organic and inorganic osmolytes from SH-SY5Y cells does not occur by a common mechanism. This observation may ultimately be of relevance to our understanding of the different roles played by the two classes of osmolytes in cell volume regulation.



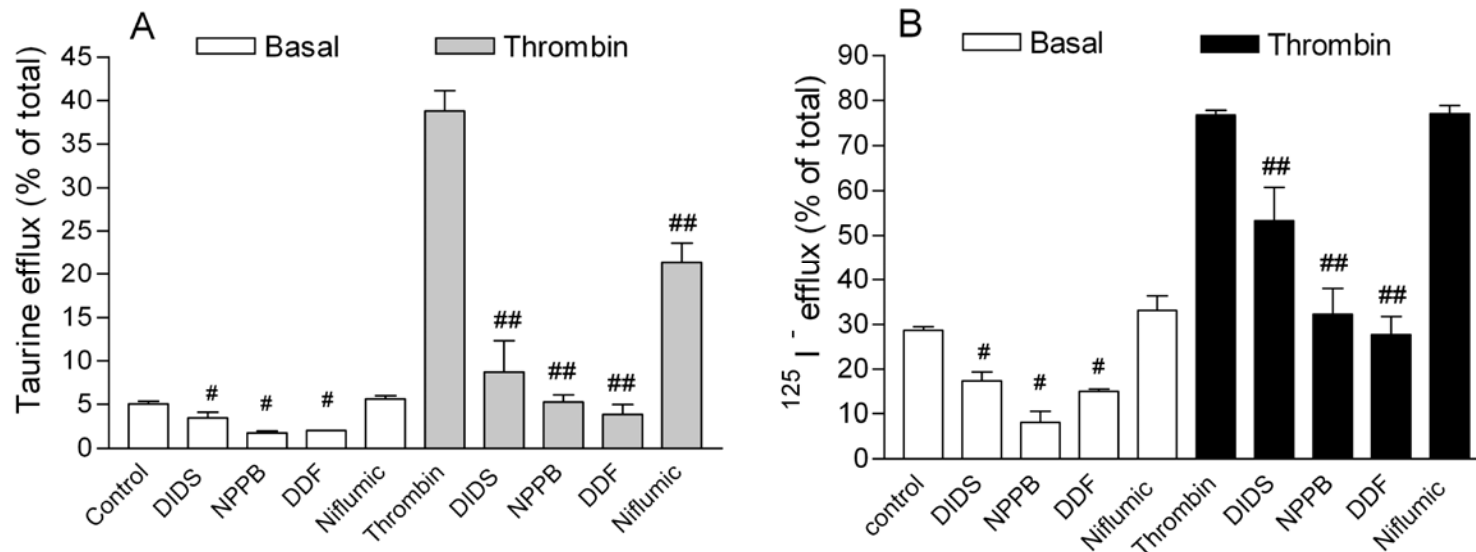
**Figure 3.1. Time course of basal- and thrombin-stimulated efflux of taurine and  $^{125}\text{I}^-$  from human SH-SY5Y neuroblastoma cells.** (A) SH-SY5Y neuroblastoma cells that had been prelabeled in the presence of [ $^3\text{H}$ ]taurine or  $^{125}\text{I}^-$  were washed two or three times respectively with 2 ml of isotonic buffer A. The cells were then incubated in 230 mOsm buffer A in the presence or absence of 1.25 nM thrombin. Reactions were terminated at the times indicated and radioactivity measured (basal release, dotted lines; thrombin-stimulated release, solid lines). Unlike taurine,  $^{125}\text{I}^-$  was not tightly retained by SH-SY5Y cells and the isotonic release of this tracer under basal conditions was approximately 50% of that observed at 230 mOsm after a 5 min incubation and 75% at longer time intervals (10-15 min). Results are expressed as taurine or  $^{125}\text{I}^-$  efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 3 independent experiments. (B) Net thrombin-mediated release (i.e. thrombin-stimulated minus basal) of taurine and  $^{125}\text{I}^-$



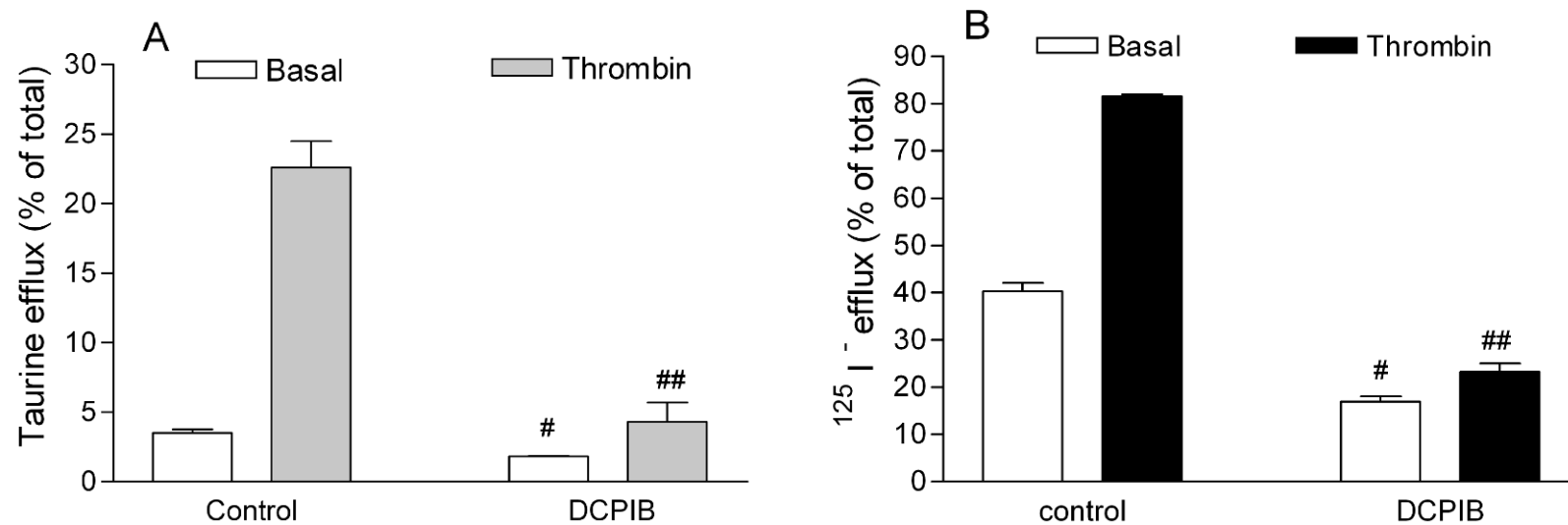
**Figure 3.2. Thrombin enhances taurine and <sup>125</sup>I<sup>-</sup> efflux from SH-SY5Y neuroblastoma cells via the PAR-1 subtype.** Cells that had been pre-labeled with (A) [<sup>3</sup>H]taurine or (B) <sup>125</sup>I<sup>-</sup> were first washed in isotonic buffer A and then incubated for 5 min in 230 mOsM buffer A in the presence or absence of either thrombin (1.25 nM) or synthetic peptides specific for PAR-1 (TFLLRN; 100 μM), PAR-3 (TFRGAP; 500 μM) or PAR-4 (GYPGKF; 500 μM) subtypes. Results are expressed as taurine or <sup>125</sup>I<sup>-</sup> efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 3 independent experiments. \*\*, Different from basal control, p < 0.01 (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).



**Figure 3.3. Basal- and thrombin-stimulated release of taurine and <sup>125</sup>I<sup>-</sup> as a function of osmolarity.** Cells prelabeled with (A) [<sup>3</sup>H]taurine or (B) <sup>125</sup>I<sup>-</sup> were first washed in isotonic buffer A and then incubated for 5 min in buffers at the osmolarities indicated in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Results are expressed as taurine or <sup>125</sup>I<sup>-</sup> efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 independent experiments. #, Different from <sup>125</sup>I<sup>-</sup> release observed in cells incubated in isotonic buffer A (340 mOsM), p < 0.05 (by one-way ANOVA followed by Dunnett's multiple comparison test). \*\*, Different from basal release, p < 0.05 (by Student's paired t-test).

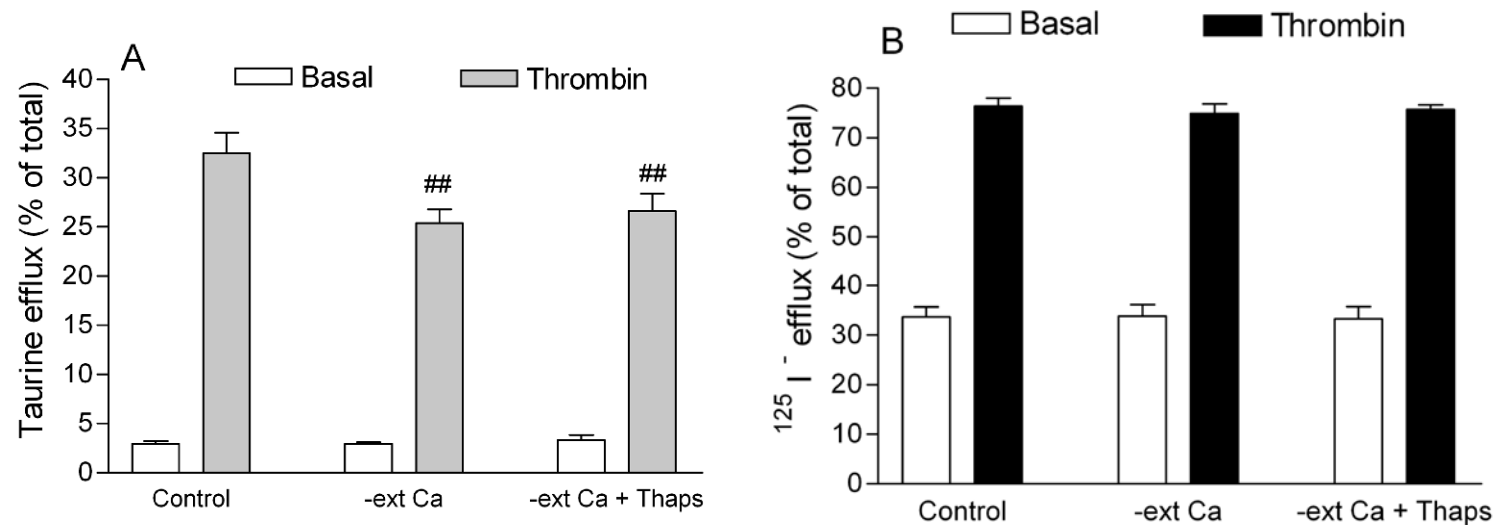


**Figure 3.4. Inhibition of basal- and thrombin-stimulated efflux of taurine and <sup>125</sup>I<sup>-</sup> by broad spectrum anion channel blockers.** Cells that had been prelabeled with (A) [<sup>3</sup>H]taurine or (B) <sup>125</sup>I<sup>-</sup> were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsM) with 200 μM DIDS, 100 μM NPPB, 100 μM dideoxyforskolin (DDF) or 100 μM niflumic acid, in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux of taurine and <sup>125</sup>I<sup>-</sup> monitored. Results are expressed as taurine or <sup>125</sup>I<sup>-</sup> efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3-5 independent experiments. #, Different from control basal, p<0.05 and ##, different from efflux in the presence of thrombin alone, p<0.05 (by Student's paired t-test).

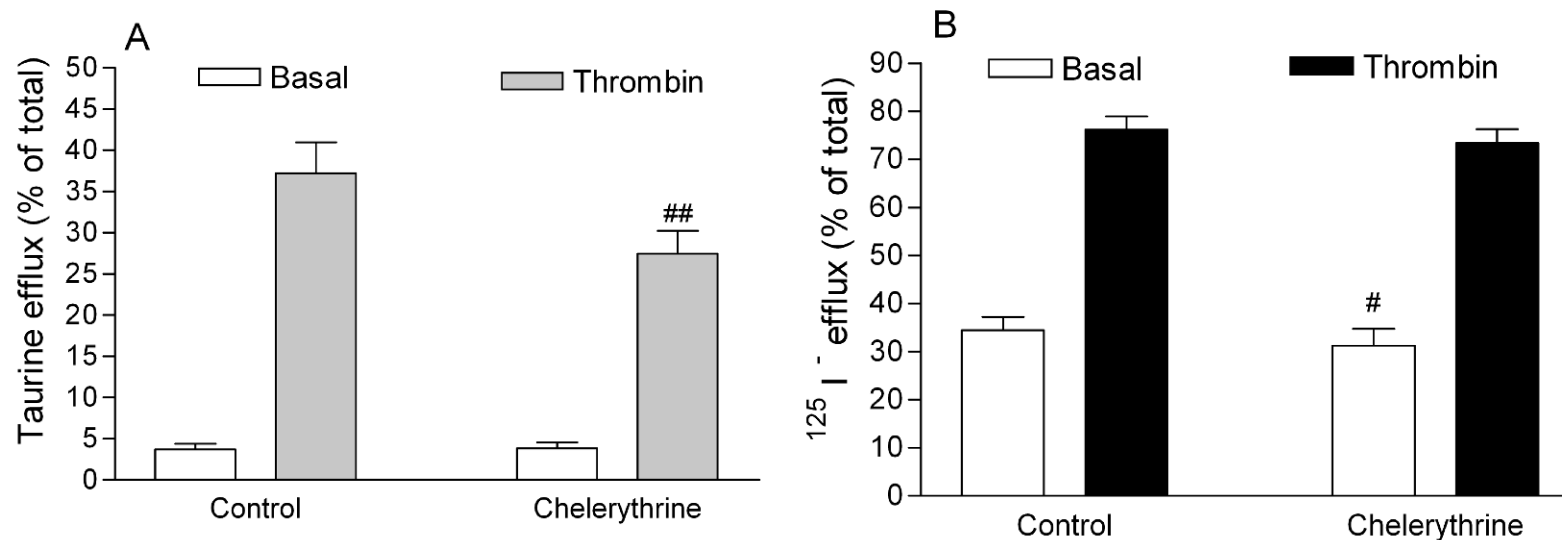


**Figure 3.5. DCPIB inhibits the basal and thrombin-stimulated efflux of both taurine and  $^{125}\text{I}^-$  efflux.** Cells prelabeled with (A) [ $^3\text{H}$ ]taurine or (B)  $^{125}\text{I}^-$  were first pretreated with 20  $\mu\text{M}$  DCPIB in isotonic buffer A for 10 min before incubation in hypotonic buffer A (230 mOsm) containing 20  $\mu\text{M}$  DCPIB in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux of taurine and  $^{125}\text{I}^-$  monitored. Results are expressed as taurine or  $^{125}\text{I}^-$  efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 4-5 independent experiments. #, Different from control basal efflux,  $p < 0.01$  (taurine);  $p < 0.001$  ( $^{125}\text{I}^-$ ) and ##, different from control thrombin-stimulated efflux,  $p < 0.01$  (taurine);  $p < 0.001$  ( $^{125}\text{I}^-$ ) (by Student's paired t-test).

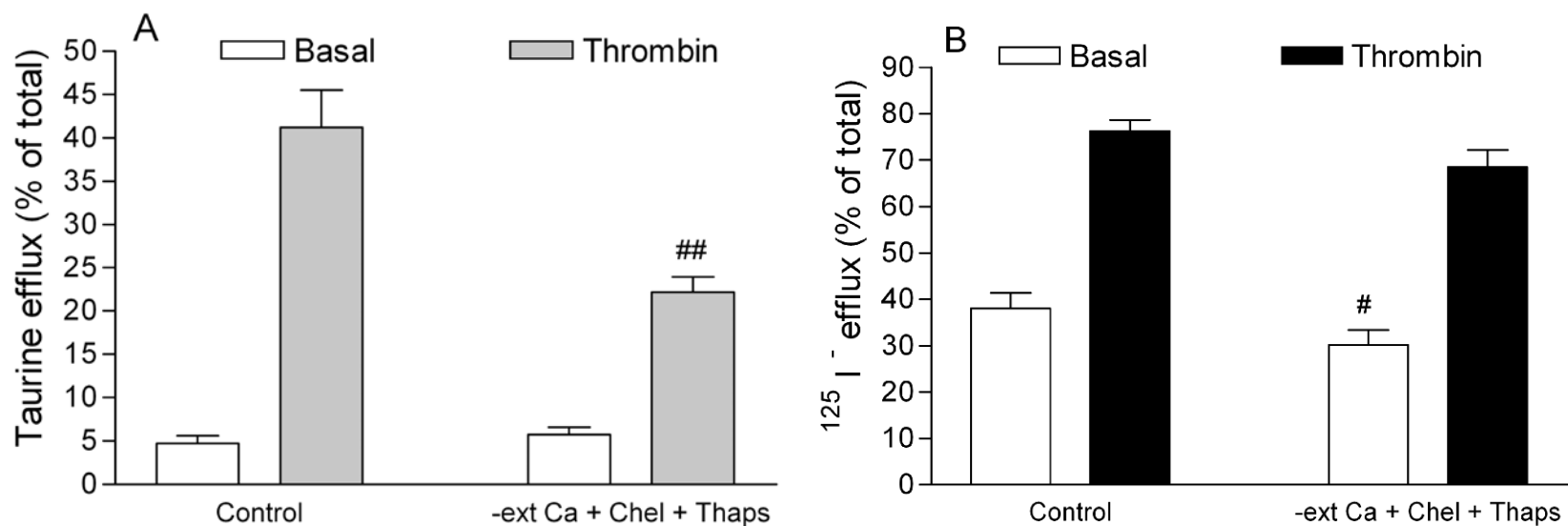




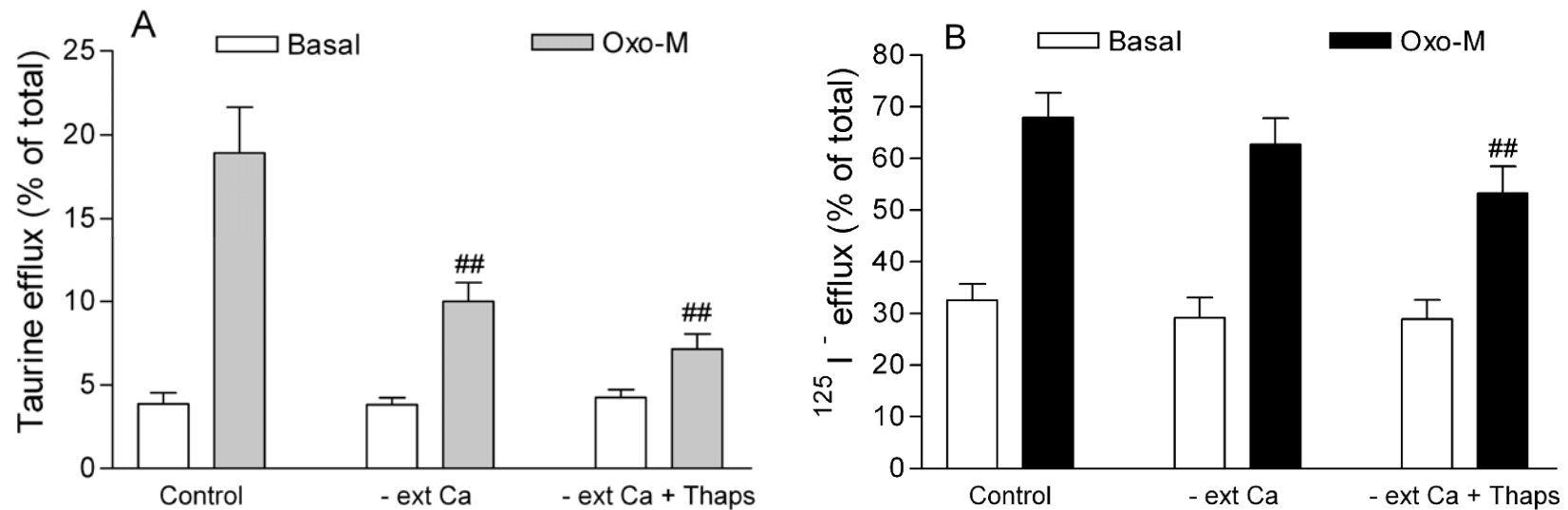
**Figure 3.6. The role of extra- and intracellular  $\text{Ca}^{2+}$  in thrombin-stimulated efflux of taurine and  $^{125}\text{I}^-$ .** (A) Cells that had been prelabeled overnight with [ $^3\text{H}$ ]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsm) in the absence (-ext Ca:  $\text{Ca}^{2+}$  was omitted from buffer and 50  $\mu\text{M}$  EGTA added) or presence of extracellular  $\text{Ca}^{2+}$ . Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. In some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 1  $\mu\text{M}$  thapsigargin (Thaps) prior to the 5 min incubation in hypotonic buffer. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 4 independent experiments. <sup>##</sup>, Different from thrombin-stimulated efflux under control conditions,  $p < 0.01$  (by one-way ANOVA followed by Dunnett's multiple comparisons test). (B) Cells prelabeled with  $^{125}\text{I}^-$  were treated as described in (A). Results are expressed as  $^{125}\text{I}^-$  efflux (percent of total soluble radioactivity), and are the means  $\pm$  S.E.M. for 4 independent experiments.



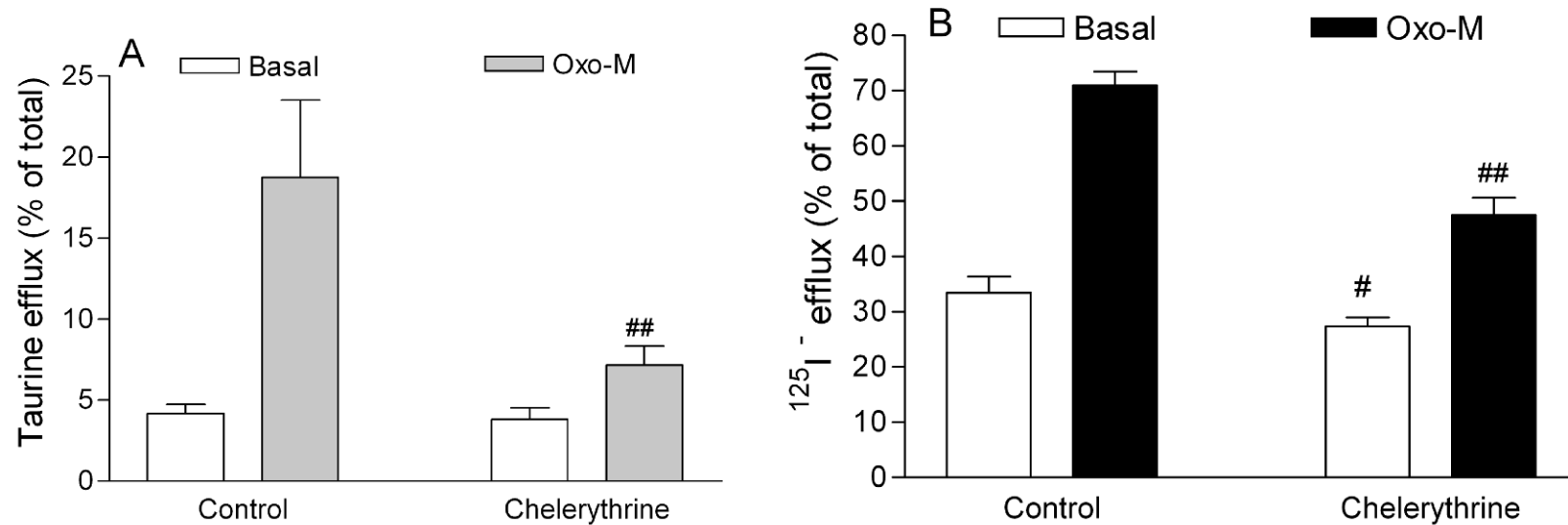
**Figure 3.7. Inhibition of the thrombin-stimulated efflux of taurine, but not of <sup>125</sup>I<sup>-</sup>, by chelerythrine.** Cells prelabeled with (A) [<sup>3</sup>H]taurine or (B) <sup>125</sup>I<sup>-</sup> were pretreated with 10 μM chelerythrine in isotonic buffer A for 15 min before incubation in hypotonic buffer A (230 mOsm) in either the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux monitored. Results are expressed as taurine or <sup>125</sup>I<sup>-</sup> efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 4 independent experiments. #, Different from control basal efflux p < 0.05 (<sup>125</sup>I<sup>-</sup>) and ##, different from thrombin-stimulated efflux under control conditions, p < 0.01 (taurine) (by Student's paired t-test).



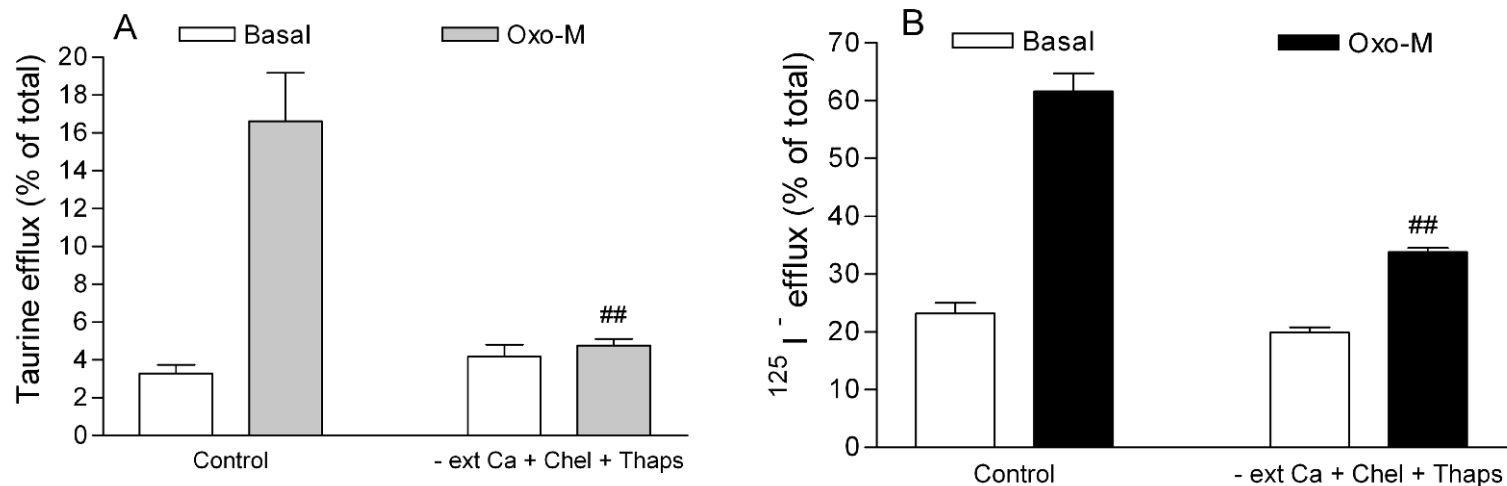
**Figure 3.8. Depletion of  $\text{Ca}^{2+}$  and inhibition of PKC only attenuates thrombin-stimulated taurine efflux.** Cells prelabeled with (A) [ $^3\text{H}$ ]taurine or (B)  $^{125}\text{I}^-$  were first preincubated for 15 min in the absence (control) or presence of 10  $\mu\text{M}$  chelerythrine and 1  $\mu\text{M}$  thapsigargin (Thaps) in isotonic buffer A. The medium was then aspirated and replaced with 230 mOsM buffer A, that either contained  $\text{Ca}^{2+}$  (control) or had  $\text{Ca}^{2+}$  omitted and 50  $\mu\text{M}$  EGTA, 1  $\mu\text{M}$  thapsigargin and 10  $\mu\text{M}$  chelerythrine added. Reactions were then allowed to proceed for 5 min in the absence (open bars) or presence (closed bars) of 1.25 nM thrombin. Results are expressed as taurine or  $^{125}\text{I}^-$  efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 4-7 independent experiments. #, Different from control basal efflux,  $p < 0.05$  ( $^{125}\text{I}^-$ ) and ##, different from thrombin-stimulated efflux under control conditions,  $p < 0.05$  (taurine) (by Student's paired t-test).



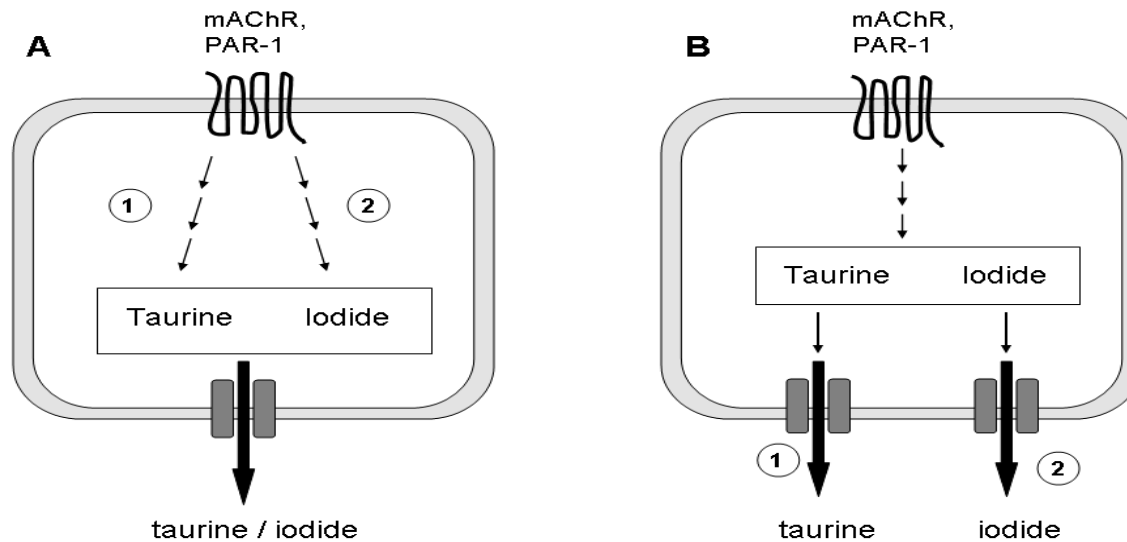
**Figure 3.9. The role of extra- and intracellular  $\text{Ca}^{2+}$  in Oxo-M-stimulated efflux of taurine and  $^{125}\text{I}^-$ .** (A) Cells that had been prelabeled overnight with [ $^3\text{H}$ ]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsm) in either the absence (-ext Ca:  $\text{Ca}^{2+}$  was omitted from buffer and 50  $\mu\text{M}$  EGTA added) or presence of extracellular  $\text{Ca}^{2+}$ . Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (filled bars) of 100  $\mu\text{M}$  Oxo-M. In some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 1  $\mu\text{M}$  thapsigargin (Thaps) prior to the 5 min incubation in hypotonic buffer. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 3 independent experiments. <sup>##</sup>, Different from Oxo-M-stimulated efflux under control conditions,  $p < 0.01$  (by one-way ANOVA followed by Dunnett's multiple comparisons test). (B) Cells prelabeled with  $^{125}\text{I}^-$  were treated as described in (A). Results are expressed as  $^{125}\text{I}^-$  efflux (percent of total soluble radioactivity), and are the means  $\pm$  S.E.M. for 5 independent experiments. <sup>##</sup>, Different from Oxo-M-stimulated efflux under control conditions,  $p < 0.01$  (by one-way ANOVA followed by Dunnett's multiple comparisons test).



**Figure 3.10. Inhibition of Oxo-M-stimulated efflux of taurine and  $^{125}\text{I}^-$  by chelerythrine.** Cells prelabeled with (A) [ $^3\text{H}$ ]taurine or (B)  $^{125}\text{I}^-$  were pretreated with 10  $\mu\text{M}$  chelerythrine in isotonic buffer A for 15 min before incubation in hypotonic buffer A (230 mOsm) in the absence (open bars) or presence (filled bars) of 100  $\mu\text{M}$  Oxo-M. Reactions were terminated after 5 min and efflux monitored. Results are expressed as taurine or  $^{125}\text{I}^-$  efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M for 4 independent experiments. #, Different from basal efflux under control conditions,  $p < 0.05$  ( $^{125}\text{I}^-$ ) and ##, different from thrombin-stimulated efflux under control conditions,  $p < 0.05$  (taurine and  $^{125}\text{I}^-$ ) (by Student's paired t-test).



**Figure 3.11. Depletion of  $\text{Ca}^{2+}$  and inhibition of PKC abolishes Oxo-M-stimulated taurine efflux and attenuates that of  $^{125}\text{I}^-$ .** Cells prelabeled with [ $^3\text{H}$ ]taurine (A) or  $^{125}\text{I}^-$  (B) were first preincubated for 15 min in the absence (control) or presence of 10  $\mu\text{M}$  chelerythrine and 1  $\mu\text{M}$  thapsigargin (Thaps) in isotonic buffer A. The medium was then aspirated and replaced with 230 mOsM buffer A, that either contained  $\text{Ca}^{2+}$  (control) or had  $\text{Ca}^{2+}$  omitted and 50  $\mu\text{M}$  EGTA, 1  $\mu\text{M}$  thapsigargin and 10  $\mu\text{M}$  chelerythrine added. Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (closed bars) of 100  $\mu\text{M}$  Oxo-M. Results are expressed as taurine or  $^{125}\text{I}^-$  efflux (percentage of total soluble radioactivity) and are the means  $\pm$  S.E.M. for 4 independent experiments. **##**, Different from Oxo-M-stimulated efflux under control conditions,  $p < 0.05$  (taurine and  $^{125}\text{I}^-$ ) (by Student's paired t-test).



**Figure 3.12. Potential mechanisms that may account for differences in Ca<sup>2+</sup> and PKC requirements for receptor-mediated release of osmolytes in SH-SY5Y cells.** (A) PAR-1 and mAChRs, in a receptor-specific manner, can activate at least two different signal transduction pathways linked to osmolyte release. Signal transduction pathway ① is dependent upon Ca<sup>2+</sup> availability and PKC activity and is *primarily* linked to taurine efflux. Signal transduction pathway ② is independent of Ca<sup>2+</sup> and PKC activity and is *primarily* coupled to iodide efflux. The efflux of both taurine and iodide is mediated via a common membrane channel that is permeable to both organic osmolytes and iodide. The majority of taurine efflux elicited by both PAR-1 and mAChRs (and a fraction of iodide release following mAChR activation) is mediated by pathway ①. In contrast, all of the iodide efflux elicited by PAR-1 receptors, and a significant fraction of that resulting from mAChR activation, is mediated by pathway ②. (B) The receptor-specific release of taurine and iodide from the cell occurs via distinct membrane channels. Channel ① is *primarily* permeable to taurine and is regulated by Ca<sup>2+</sup> and PKC. In contrast, Channel ② *primarily* mediates the release of iodide and is independent of both Ca<sup>2+</sup> and PKC activity. Channel ① mediates the majority of taurine release resulting from activation of both PAR-1 and mAChRs (and a fraction of iodide release resulting from mAChR activation) whereas Channel ② mediates all of the iodide efflux elicited by the PAR-1 receptor and a fraction of that following mAChR activation. It remains possible that both mechanisms (A) and (B) operate concurrently.

## References

- Abdullaev IF, Rudkouskaya A, Schools GP, Kimelberg HK and Mongin AA (2006) Pharmacological comparison of swelling-activated excitatory amino acid release and  $\text{Cl}^-$  currents in cultured rat astrocytes. *J Physiol* 572:677-689.
- Banderali U and Roy G (1992) Activation of  $\text{K}^+$  and  $\text{Cl}^-$  channels in MDCK cells during volume regulation in hypotonic media. *J Membr Biol* 126:219-234.
- Best L, Yates AP, Decher N, Steinmeyer K and Nilius B (2004) Inhibition of glucose-induced electrical activity in rat pancreatic beta-cells by DCPIB, a selective inhibitor of volume-sensitive anion currents. *Eur J Pharmacol* 489:13-19.
- Cardin V, Lezama R, Torres-Marquez ME and Pasantes-Morales H (2003) Potentiation of the osmosensitive taurine release and cell volume regulation by cytosolic  $\text{Ca}^{2+}$  rise in cultured cerebellar astrocytes. *Glia* 44:119-128.
- Carton I, Trouet D, Hermans D, Barth H, Aktories K, Droogmans G, Jorgensen NK, Hoffman EK, Nilius B and Eggermont J (2002) RhoA exerts a permissive effect on volume-regulated anion channels in vascular endothelial cells. *Am J Physiol* 283: C115-C125.
- Cheema TA, Ward CE and Fisher SK (2005) Subnanomolar concentrations of thrombin enhance the volume-sensitive efflux of taurine from human 1321N1 astrocytoma cells. *J Pharmacol Exp Ther* 315:755-763.
- Davis-Amaral EM, Musch MW and Goldstein L (1996) Chloride and taurine effluxes occur by different pathways in skate erythrocytes. *Am J Physiol* 271:R1544-1549.
- Decher N, Lang HJ, Nilius B, Bruggemann A, Busch AE and Steinmeyer K (2001) DCPIB is a novel selective blocker of  $I_{(\text{Cl},\text{swell})}$  and prevents swelling-induced shortening of guinea-pig atrial action potential duration. *Br J Pharmacol* 134:1467-1479.
- Fisher SK, Domask LM and Roland RM (1989) Muscarinic receptor regulation of cytoplasmic  $\text{Ca}^{2+}$  concentrations in human SK-N-SH neuroblastoma cells:  $\text{Ca}^{2+}$  requirements for phospholipase C activation. *Mol Pharmacol* 35:195-204.
- Heacock AM, Kerley D, Gurda GT, VanTroostenberghe AT and Fisher SK (2004) Potentiation of the osmosensitive release of taurine and D-aspartate from SH-SY5Y neuroblastoma cells after activation of M3 muscarinic cholinergic receptors. *J Pharmacol Exp Ther* 311:1097-1104



- Heacock AM, Dodd MS and Fisher SK (2006) Regulation of volume-sensitive osmolyte efflux from human SH-SY5Y neuroblastoma cells following activation of lysophospholipid receptors. *J Pharmacol Exp Ther* 317:685-693.
- Huxtable RJ (1992) Physiological actions of taurine. *Physiol Rev* 72:101-163.
- Jackson PS and Strange K (1993) Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. *Am J Physiol* 265:C1489-1500.
- Junankar PR, Karjalainen A and Kirk K (2002) The role of P2Y1 purinergic receptors and cytosolic  $Ca^{2+}$  in hypotonically activated osmolyte efflux from a rat hepatoma cell line. *J Biol Chem* 277:40324-40334.
- Kimelberg HK (2000) Cell volume in the CNS: regulation and implications for nervous system function and pathology. *The Neuroscientist* 6:14-25.
- Lambert DG and Nahorski SR (1990) Muscarinic-receptor-mediated changes in intracellular  $Ca^{2+}$  and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY5Y. *Biochem J* 265:555-562.
- Lambert IH (2004) Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. *Neurochem Res* 29:27-63.
- Lambert IH and Hoffmann EK (1994) Cell swelling activates separate taurine and chloride channels in Ehrlich mouse ascites tumor cells. *J Membr Biol* 142:289-298.
- Lang F, Busch GL and Volkl H (1998) The diversity of volume regulatory mechanisms. *Cell Physiol Biochem* 8:1-45.
- Large WA and Wang Q (1996) Characteristics and physiological role of the  $Ca^{2+}$ -activated  $Cl^-$  conductance in smooth muscle. *Am J Physiol* 271:C435-454.
- Loveday D, Heacock AM and Fisher SK (2003) Activation of muscarinic cholinergic receptors enhances the volume-sensitive efflux of myo-inositol from SH-SY5Y neuroblastoma cells. *J Neurochem* 87:476-486.
- Manolopoulos GV, Prenen J, Droogmans G and Nilius B (1997) Thrombin potentiates volume-activated chloride currents in pulmonary artery endothelial cells. *Pflugers Arch* 433:845-847

- Mongin AA and Kimelberg HK (2002) ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. *Am J Physiol Cell Physiol* 283:C569-578.
- Mongin AA and Kimelberg HK (2005) ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple  $\text{Ca}^{2+}$ -sensitive mechanisms. *Am J Physiol Cell Physiol* 288:C204-213.
- Moran J, Morales-Mulia S, Hernandez-Cruz A and Pasantes-Morales H (1997) Regulatory volume decrease and associated osmolyte fluxes in cerebellar granule neurons are calcium independent. *J Neurosci Res* 47:144-154.
- Nilius B and Droogmans G (2003) Amazing chloride channels: an overview. *Acta Physiol Scand* 177:119-147.
- Pasantes-Morales H, Cardin V and Tuz K (2000) Signaling events during swelling and regulatory volume decrease. *Neurochem Res* 25:1301-1314.
- Pasantes-Morales H, Franco R, Ordaz B and Ochoa LD (2002) Mechanisms counteracting swelling in brain cells during hyponatremia. *Arch Med Res* 33:237-244.
- Pederson SF, Beisner KH, Hougaard C, Willumsen BM, Lambert IH and Hoffman EK. (2002). Rho family GTP binding proteins are involved in the regulatory volume decrease process in NIH3T3 mouse fibroblasts. *J Physiol* 541:779-796.
- Sanchez-Olea R, Morales M, Garcia O and Pasantes-Morales H (1996)  $\text{Cl}^-$  channel blockers inhibit the volume-activated efflux of  $\text{Cl}^-$  and taurine in cultured neurons. *Am J Physiol* 270:C1703-1708.
- Shennan DB, Cliff MJ and Hawkins P (1996) Volume-sensitive taurine efflux from mammary tissue is not obliged to utilize volume-activated anion channels. *Biosci Rep* 16:459-465.
- Shennan DB and Thomson J (2000) Further evidence for the existence of a volume-activated taurine efflux pathway in rat mammary tissue independent from volume-sensitive  $\text{Cl}^-$  channels. *Acta Physiol Scand* 168:295-299.
- Stutzin A, Torres R, Oporto M, Pacheco P, Eguiguren AL, Cid LP and Sepulveda FV (1999) Separate taurine and chloride efflux pathways activated during regulatory volume decrease. *Am J Physiol* 277:C392-402.
- Thompson AK and Fisher SK (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated

phosphoinositide turnover in two neuroblastomas: methodological considerations. *J Pharmacol Exp Ther* 252:744-752.

Tomassen SF, Fekkes D, de Jonge HR and Tilly BC (2004) Osmotic swelling-provoked release of organic osmolytes in human intestinal epithelial cells. *Am J Physiol Cell Physiol* 286:C1417-1422.

Upadhyay A, Jaber BL and Madias NE (2006) Incidence and prevalence of hyponatremia. *Am J Med* 119:S30-35.

## CHAPTER 4

### CHOLESTEROL REGULATES VOLUME-SENSITIVE OSMOLYTE EFFLUX FROM HUMAN SH-SY5Y NEUROBLASTOMA CELLS FOLLOWING RECEPTOR ACTIVATION

#### Summary

The ability of cholesterol to modulate receptor-mediated increases in the volume-dependent release of the organic osmolyte, taurine, has been examined. Depletion of cholesterol from SH-SY5Y neuroblastoma by pre-incubation of the cells with 5 mM methyl- $\beta$ -cyclodextrin (CD) for 10 min resulted in a 40-50% reduction in cholesterol and an enhancement of the ability of proteinase-activated -1 (PAR-1), muscarinic cholinergic (mAChR) and sphingosine 1-phosphate receptors to stimulate taurine efflux, when monitored under hypoosmotic conditions. Basal (swelling-induced) release of taurine was also enhanced by cholesterol depletion, but less markedly. Both basal- and receptor-mediated increases in taurine efflux were mediated via a volume-sensitive organic osmolyte and anion channel in control and cholesterol-depleted cells. Studies with the PAR-1 and mAChR receptor subtypes indicated that the stimulatory effect of CD pretreatment could be reversed by incubation of the cells with either CD:cholesterol or CD:epicholesterol donor complexes and that cholesterol depletion increased agonist efficacy, but not potency. The ability of cholesterol depletion to promote the PAR-1 receptor-mediated stimulation of osmolyte

release was most pronounced under conditions of isotonicity or mild hypotonicity. In contrast to CD pretreatment, preincubation of the cells with either cholesterol oxidase, or sphingomyelinase, conditions under which lipid microdomains are also disrupted, had no effect on either basal- or receptor-stimulated taurine efflux. Taken together, the results suggest that cholesterol regulates receptor-mediated osmolyte release via its effects on the biophysical properties of the plasma membrane, rather than its presence in lipid microdomains.

### **Introduction**

Volume regulation is a fundamental homeostatic mechanism used by cells and is of particular importance to the central nervous system because of restrictions of the skull. Even minor increases in brain volume can have adverse effects on cell-cell signaling events and more severe brain swelling, as may occur during episodes of hyponatremia, and can lead to cerebral anoxia, ischemia and ultimately death due to cardiac and respiratory arrest (Pasantes-Morales et al., 2000). In response to hypotonic stress, both neural and non-neural cells regulate their volume through the efflux of  $K^+$ ,  $Cl^-$  and “compatible” organic osmolytes such as taurine, glutamate or myo-inositol. Loss of these osmolytes results in the exit of obligated water thereby restoring cell volume, a process known as regulatory volume decrease. Organic osmolytes and  $Cl^-$  are released from cells via a volume-sensitive organic osmolyte and anion channel (VSOAC), a channel that has been extensively characterized both

electrophysiologically and pharmacologically, although its molecular structure remains unknown (Lang et al., 1998; Nilius and Droogmans, 2003).

Given the central role that maintenance of volume plays in the physiology of neural cells, mechanisms involved in its regulation assume major significance. In this context, we and others have recently demonstrated that the volume-dependent release of osmolytes from a variety of neural cells can be dramatically enhanced following activation of certain G protein-coupled receptors (Mongin and Kimelberg, 2002, 2005; Heacock et al., 2004, 2006; Cheema et al., 2005, 2007; Ramos-Mandujano et al., 2007). Receptor activation not only increases the extent of osmolyte release, but it also lowers the threshold osmolarity ('set-point') at which osmolytes are released, thereby permitting cells to respond to small, physiologically relevant, reductions in osmolarity. This observation raises the possibility that, in vivo, receptor activation may be a pre-requisite for the efficient volume regulation of cells. Although volume-dependent osmolyte release from cells is mediated via a VSOAC under both basal- (swelling-induced) and receptor-stimulated conditions (Abdullaev et al., 2006; Cheema et al., 2007), separate mechanisms underlie these two responses, as is evident from their differential sensitivities to  $Ca^{2+}$  availability and activation of protein kinase C (Mongin and Kimelberg, 2005; Heacock et al., 2006).

Cholesterol availability represents an additional means whereby the activity of VSOAC may be regulated. In some non-neural cells, depletion of cholesterol facilitates the activity of VSOAC in response to hypotonicity, an effect that is reversed by increases in the cholesterol content of the cells (Levitan et al.,

2000; Romanenko et al., 2004; Klausen et al., 2006; Byfield et al., 2006, Lim et al., 2006). As previously observed for receptor activation, cholesterol depletion is reported to optimally facilitate basal VSOAC activity under conditions of limited reductions in osmolarity. However, whether cholesterol availability can further regulate receptor-stimulated osmolyte release under physiologically relevant conditions has yet to be addressed. In addition, the mechanism(s) underlying cholesterol regulation of basal VSOAC activity has not been established. In endothelial cells, cholesterol dependence of VSOAC activity has been attributed to changes in membrane fluidity and/or a negative regulation of VSOAC by segregation of the channel into membrane lipid domains such as rafts or caveolae (Romanenko et al., 2004; Byfield et al., 2006). In contrast, in other cell types, VSOAC activity appears to be dependent upon the integrity of caveolae (Trouet et al., 1999, 2001; Ullrich et al., 2006). Since multiple receptor subtypes, and associated signaling proteins have been localized to caveolae and/or lipid rafts (Allen et al., 2007), it is conceivable that cholesterol availability may differentially influence swelling-induced and receptor-activated VSOAC activity.

In the present study, we have addressed the possibility that cholesterol availability can regulate receptor-mediated VSOAC activity by monitoring the effects of cholesterol depletion and re-addition on volume-dependent taurine release from human SH-SY5Y neuroblastoma cells. We have previously demonstrated that these cells possess several pharmacologically distinct, receptors that couple to osmolyte efflux, including protease-activated (PAR-1), muscarinic cholinergic (mAChR) and sphingosine 1-phosphate (S1P: Heacock et

al., 2004, 2006; Cheema et al., 2007). The results indicate that cholesterol depletion, induced by methyl- $\beta$ -cyclodextrin (CD), synergistically enhances the ability of all three receptors to increase taurine efflux above basal levels and that this potentiation can be reversed by administration of cholesterol or its stereoisomer, epicholesterol. Cholesterol depletion appears to exert its most significant regulatory influence on receptor-stimulated taurine efflux under conditions of isotonicity or mild hypotonicity. Thus, in SH-SY5Y neuroblastoma cells, both cholesterol availability and receptor activation may act in concert to enable the cells to respond to small changes in osmolarity.

## Methods

**Materials.** [1,2- $^3$ H] Taurine (1.15 TBq/ml) was obtained from Amersham Biosciences (Piscataway, NJ). Thrombin, oxotremorine-M, sphingosine 1-phosphate, methyl- $\beta$ -cyclodextrin, cholesterol (5-cholesten-3 $\beta$ -ol), sphingomyelinase from *Staphylococcus aureus* and cholesterol oxidase were purchased from Sigma-Aldrich (St. Louis, MO). DCPIB was obtained from Tocris Biosciences (Ellisville, MO). Epicholesterol (5-cholesten-3 $\alpha$ -ol) was purchased from Steraloids (Newport, RI). Amplex Red Assay kit was from Molecular Probes, Inc. (Eugene, OR). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences



(Franklin Lakes, NJ). Universol was obtained from Valeant Pharmaceuticals (Costa Mesa, CA).

### **Cell culture conditions.**

Human SH-SY5Y neuroblastoma cells (passages 70-89) were grown in tissue culture flasks (75 cm<sup>2</sup>/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal bovine serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330-340 mOsM. Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was aspirated and the cells detached from the flask with a trypsin-versene mixture (Biowhittaker, MD). Cells were then resuspended in DMEM/10% fetal bovine serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates for 5-6 days. Experiments were routinely conducted on cells that had reached 70–90% confluency.

### **Measurement of taurine efflux.**

Osmolyte efflux from SH-SY5Y neuroblastoma cells was monitored essentially as previously described (Heacock et al., 2004; Cheema et al., 2007). In brief, cells were prelabeled to isotopic equilibrium with 18.5 kBq/ml of [<sup>3</sup>H]taurine at 37°C for 24 h. After prelabeling, the cells were washed twice with 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl<sub>2</sub>, 3.6 mM NaHCO<sub>3</sub>, 1mM MgCl<sub>2</sub> and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 340 mOsM). Cells were then allowed to incubate in 2 ml of hypotonic buffer A (295-200 mOsM; rendered

hypotonic by a reduction in NaCl concentration) in the absence or presence of the agonists. In some experiments, buffer A was made hypertonic (370 and 450 mOsM) by the addition of NaCl. Osmolarities of buffer A were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots (200-400  $\mu$ l) of the extracellular medium were removed and radioactivity determined after the addition of 5 ml Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 ml of ice-cold 6% (wt/vol) trichloroacetic acid. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released in the extracellular media as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay. Because SH-SY5Y cells had been labeled to isotopic equilibrium with [ $^3$ H] taurine, measurement of radioactivity in the extracellular medium reflects changes in the mass of the osmolyte. Throughout this study, "basal" release of taurine is defined as that which occurs at a specified osmolarity in the absence of an agonist.

### **Modulation of cellular cholesterol content and substitution of cholesterol with epicholesterol**

SH-SY5Y neuroblastoma were either depleted of cholesterol by incubation of the cells for 10 min at 37 $^{\circ}$ C with either 5 mM methyl- $\beta$ -cyclodextrin (CD) alone or alternatively, enriched with cholesterol by CD complexed with cholesterol

dissolved in serum-free media. Cholesterol was complexed with CD as described previously (Romanenko et al., 2004). Briefly, a small amount of cholesterol was dissolved in chloroform: methanol (1:1, by vol) in a glass tube and the solvent evaporated. Then, a 5 mM CD solution in serum-free DMEM was added to the dried cholesterol. The tube was then vortexed, sonicated and incubated overnight in a shaking bath at 37°C. CD was complexed with cholesterol at a saturating ratio of 1:8 (0.625 mM cholesterol; Christian et al., 1997). In preparation for an experiment, cells prelabeled with [<sup>3</sup>H] taurine were first depleted of cholesterol by exposing them to CD for 10 min in a humidified CO<sub>2</sub> incubator at 37°C. Cells were then washed with isotonic buffer A and exposed to a cholesterol:CD (1:8) donor complex for 1 h and then returned to the incubator. After 1 h, cells were washed once more with isotonic buffer A before measurement of taurine efflux. Control cells were treated in a similar way with serum-free DMEM. An epicholesterol: CD (1:8) complex was prepared as described for the cholesterol:CD complex above (Romanenko et al., 2002).

### **Cellular cholesterol content**

The Amplex Red-based cholesterol assay was conducted in a 96-well microplate using a 100 µl reaction volume per well. Cells were lysed with 1X reaction buffer containing 0.1 mM potassium phosphate, pH 7.4, 0.05 mM NaCl, 5 mM cholic acid and 0.1% Triton X-100. The cells were then sonicated for 20 s before 10 µl aliquots of the lysates were diluted into 40 µl of 1X reaction buffer. The cholesterol detection assay was initiated by the addition of 50 µl/well of 300 µM

Amplex Red Reagent working solution containing 2 U/ml horse radish peroxidase, 2 U/ml cholesterol oxidase and 0.2 U/ml cholesterol esterase. The reaction mixtures were incubated at 37°C for 30 min, and the fluorescence intensities were measured using a fluorescence microplate reader equipped with a filter set for excitation and emission at  $540 \pm 10$  and  $590 \pm 10$  nm, respectively (FLUOstar Optima BMG LabTech, Durham, NC). The cholesterol values were compared to a cholesterol standard curve from (0 – 8  $\mu$ g/ml). Protein assay was performed with a bicinchoninic acid protein assay reagent kit obtained from Pierce (Rockford, IL). Cholesterol values quoted are normalized to the protein content of the cell lysates.

#### **Measurement of Phosphoinositide Turnover.**

To monitor phosphoinositide turnover, SH-SY5Y cells that had been prelabeled with 111 KBq/ml of [ $^3$ H]inositol for 48 h were incubated in hypotonic buffer A (230 mOsm) that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher, 1990).

#### **Data Analysis.**

Experiments were performed in triplicate and repeated at least three times. Values quoted are given as means  $\pm$  S.E.M. for the number (n) of independent experiments indicated. A two-tailed Student's t test (paired or unpaired) was used to evaluate differences between two experimental groups (level of significance, p

< 0.05). Ordinary or repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC<sub>50</sub> values were obtained using Prism 4.0a (GraphPad Software Inc., San Diego, CA).

## Results

**Cholesterol depletion enhances both basal- and thrombin-stimulated taurine efflux.** Methyl- $\beta$ -cyclodextrin (CD), a water-soluble cyclic oligosaccharide, provides an effective and reproducible method for extracting cholesterol from a variety of cells, thereby disrupting lipid microdomains (Christian et al., 1997; Brown and London, 2000). However, since this approach has not previously been employed for SH-SY5Y cells, initial studies were directed at determining the optimal conditions for cholesterol extraction while limiting the cells to CD exposure, as recommended by Zidovetzki and Levitan (2007). When SH-SY5Y cells were pretreated with increasing concentrations of CD (1-5 mM) for 10 min at 37°C, a dose-dependent decrease in total cholesterol content was observed (Fig. 4.1A). More prolonged incubation of the cells with CD resulted in cell rounding and a loss of morphology. In SH-SY5Y cells, total cellular cholesterol (predominantly non-esterified) was  $17.0 \pm 3.0$   $\mu\text{g}/\text{mg}$  of protein (n=10). A maximum reduction in cholesterol content (43%) was observed following pretreatment of the cells with 5 mM CD for 10 min. Both basal (swelling-activated) and thrombin-stimulated taurine efflux monitored under hypotonic conditions (230 mOsm), were significantly enhanced by depletion of

cholesterol. A significant effect on basal taurine release was observed at 3 mM CD with a maximum effect observed at a 5 mM concentration. Under the latter conditions, efflux increased from 4.5% to 8.7% of the total taurine pool, a net increase of 4.2% (Fig 4.1B). CD pretreatment also significantly potentiated thrombin-stimulated taurine release at all concentrations of CD tested (1–5 mM) with a maximum observed at CD concentrations >2 mM. Following pretreatment of the cells with 5 mM CD, taurine efflux increased from 46% to 56% of the total taurine pool, a net increase in the efflux of 10% (Fig. 4.1C). In subsequent experiments a 10 min pretreatment with 5 mM CD was routinely utilized so that changes in both basal- and receptor-stimulated taurine efflux could be readily evaluated.

**Time-course of volume-dependent taurine efflux following cholesterol depletion.** When SH-SY5Y cells that had been prelabeled with [<sup>3</sup>H]taurine were exposed to hypotonic buffer A (230 mOsM; ~30% reduction in osmolarity), there was a time-dependent release of the radiolabeled amino acid from the cells, as previously reported (Cheema et al., 2007). This basal release was enhanced by ~2 fold (2.8% to 7.0% of the total taurine pool) when the cells were pretreated with 5 mM CD for 10 min at 37°C (Fig. 4.2). Pre-treatment of cells with CD also significantly enhanced the ability of thrombin (0.25U/ml, equivalent to 1.25 nM) to facilitate taurine efflux at all time points examined (Fig. 4.2). Since the CD-mediated increases in the magnitude of both basal-and receptor-stimulated

release of taurine were maximal at 5 min of incubation or thereafter, osmolyte efflux was subsequently routinely monitored following a 5 min incubation.

**Cholesterol depletion facilitates taurine efflux elicited by activation of multiple receptors.** We have previously demonstrated that SH-SY5Y cells express mAChR, PAR-1 and S1P receptors, the activation of which significantly increase taurine release during hypotonic stress (Loveday et al., 2003; Heacock et al., 2006; Cheema et al., 2007). To determine whether the ability of cholesterol depletion to enhance taurine release is receptor-specific, the effect of CD pretreatment on the ability of all three receptors to stimulate taurine efflux was evaluated. It was observed that CD pretreatment facilitated the ability of thrombin, Oxo-M and S1P to enhance the volume-dependent release of taurine (net increases in efflux of 13.4%, 14.8% and 19.9% of the total taurine pool, respectively). In contrast, the corresponding value for the net increase in basal release of taurine observed following cholesterol depletion was 5.6% (Fig. 4.3). Moreover, the increase in receptor-mediated efflux of taurine from cholesterol-depleted cells was greater than that due to the increase in basal release alone, i.e. a synergistic, rather than additive, enhancement of receptor-mediated osmolyte efflux was observed for all three receptors ( $p < 0.01$ ; paired Student's t-test). Because previous studies of SH-SY5Y cells have extensively characterized the coupling of PAR-1 and mAChRs to volume-dependent taurine efflux (Heacock et al., 2004, 2006; Cheema et al., 2007), in the current study emphasis has been placed on monitoring the effects of cholesterol depletion and repletion

on the activity of these receptor subtypes. In addition, the effects (if any) of CD on signal transduction events can readily be assessed by monitoring mAChR-stimulated phosphoinositide turnover in these cells.

**CD pretreatment increases agonist efficacy for taurine efflux but not agonist potency.** To determine whether cholesterol depletion enhances agonist potency and/or efficacy, dose-response curves for taurine efflux were constructed for thrombin and Oxo-M, for both control cells and those pretreated with CD. Cholesterol depletion increased agonist efficacy over the range of concentrations of thrombin or Oxo-M tested. However, agonist potency was unaffected by CD pretreatment. Thus, EC<sub>50</sub> values were 105 pM and 60 pM for thrombin and 0.9 μM and 1.9 μM for Oxo-M in the absence or presence of CD pretreatment, respectively (Fig. 4.4 A, B).

**Blockade of VSOAC with DCPIB attenuates the CD-induced facilitation of taurine release.** DCPIB, an agent that is considered highly selective for VSOAC (Decher et al., 2001; Best et al., 2004; Abdullaev et al., 2006), was examined for its ability to inhibit the CD-induced increases in taurine efflux. Inclusion of 20 μM DCPIB significantly inhibited basal taurine efflux monitored under both control and CD-pretreated conditions (56% and 67% inhibition, respectively). Similarly, DCPIB also inhibited thrombin-stimulated taurine efflux monitored in either control or CD-pretreated cells (94% and 76% inhibition, respectively: Fig. 4.5).



**Osmolarity dependence of basal- and thrombin-stimulated taurine efflux under conditions of cholesterol depletion.** Previously we have demonstrated that receptor-mediated enhancement of osmolyte release is dependent on the degree of hypo-osmotic stress in SH-SY5Y cells (Heacock et al., 2004, 2006; Cheema et al., 2007). Thus the ability of cholesterol depletion to potentiate taurine release at different osmolarities was examined. SH-SY5Y cells were pretreated with 5 mM CD and both basal- and thrombin-stimulated release of taurine was monitored under conditions of isotonicity (340 mOsM: defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild to moderate hypotonicity (295-200 mOsM) or hypertonicity (370-450 mOsM). The basal release of taurine was not appreciably increased until the osmolarity had been reduced to 200 mOsM. Although CD pretreatment of cells further increased the basal release of taurine when monitored under conditions of hypotonicity, this effect only became significant following a 30% decrease in osmolarity (a net increase in efflux of 5.9% of the total taurine pool at 230 mOsM). As previously reported (Cheema et al., 2007), the addition of thrombin to control cells resulted in a significant increase in taurine efflux under either mild or moderate hypotonic conditions (Fig. 4.6A). CD pretreatment of the cells further enabled thrombin to enhance taurine efflux under isotonic, as well as mild to severe hypotonic, conditions. However, the stimulatory effect of cholesterol depletion on PAR-1-mediated taurine release was most marked under conditions of either isotonicity (340 mOsM) or mild hypotonicity (295 mOsM). Thus, although little or no increase in taurine efflux was observed following the addition of

thrombin to control cells incubated in isotonic buffer A, a significant increase in osmolyte release was obtained from CD-pretreated cells under isotonic conditions (a net increase in efflux of 5.3% of the total taurine pool). Similarly, cholesterol depletion increased the ability of thrombin to stimulate taurine release from 8.2 to 21.5% of the total taurine pool, when monitored at 295 mOsM (Fig. 4.6A). When thrombin-stimulated taurine release for cholesterol-depleted cells was calculated (as a percentage) relative to control cells, the values were 332%, 262%, 178% and 139% of control at 340, 295, 250 and 230 mOsM, respectively. The ability of cholesterol depletion to enhance thrombin-stimulated taurine efflux was reduced under conditions of mild hypertonicity (370 mOsM) and was abolished under more severely hypertonic conditions (450 mOsM: Fig. 4.6B).

**Enhancement of taurine efflux by CD is reversed by the re-addition of either cholesterol or epicholesterol.** The ability of CD pretreatment to facilitate either thrombin- or Oxo-M-mediated taurine efflux under hypoosmotic conditions could be fully reversed by preincubation of the cells for 1 h in the presence of a saturating (1:8) cholesterol: CD complex (0.625 mM cholesterol). In fact, following cholesterol administration, the efflux of taurine monitored under basal-, thrombin- and Oxo-M- stimulated conditions was significantly inhibited (61-77%) when compared to control values (Fig. 4.7A). Concurrent measurements of the cholesterol content of SH-SY5Y cells revealed that a 65% increase in cholesterol content over control cells was observed after the cells were exposed to the cholesterol:CD complex (Fig. 4.7B). The addition of a 1:8 cholesterol:CD

complex to control cells also resulted in an increase in cholesterol content and a reduction in both basal- and thrombin-stimulated taurine release (data not shown). The specificity with which cholesterol administration can reverse the stimulatory effects of CD pretreatment was further tested by determining whether epicholesterol, a stereoisomer of cholesterol, could substitute for cholesterol. Epicholesterol and cholesterol have previously been employed to discriminate between effects due to sterol-protein interactions and those due to changes in the physical properties of the membrane lipid bilayer (Zidovetzki and Levitan, 2007). As observed for cholesterol re-addition, administration of an epicholesterol: CD complex (1:8) reversed the stimulatory effects of cholesterol depletion and resulted in an inhibition of agonist- stimulated taurine efflux (25-29%), but did not inhibit basal release.

**Alterations in cholesterol content or replacement with epicholesterol do not influence the extent of mAChR-stimulated phosphoinositide turnover.** In some cells, CD pre-treatment results in an attenuation of cell signaling events (Burger et al., 2000). To evaluate the possibility that modulation of cholesterol content alters receptor signaling events within SH-SY5Y cells, basal- and mAChR-stimulated phosphoinositide turnover was monitored under control, cholesterol-depleted and cholesterol- or epicholesterol-supplemented conditions. Neither CD-pretreatment (Fig. 4.8A), nor supplementation with either cholesterol or epicholesterol, had any significant effect on either basal or mAChR- stimulated phosphoinositide turnover (Fig. 4.8 B, C).

**Pretreatment of SH-SY5Y cells with cholesterol oxidase or sphingomyelinase has no effect on basal- or receptor-stimulated taurine efflux.** In many cell types, a reduction in cholesterol content can also be achieved following incubation of the cells with cholesterol oxidase, an enzyme that converts cholesterol into 4-cholesten-3-one, a sterol which exhibits membrane fluidity characteristics similar to those of cholesterol (Gimpl et al., 1997). Preincubation of SH-SY5Y cells with cholesterol oxidase (1.5 U/ml) for 1h had no effect on basal-, mAChR- or PAR-1-stimulated taurine efflux under hypotonic conditions (Fig. 4.9A), although the cholesterol content of SH-SY5Y cells was reduced by 57% (Fig. 4.9B). Pretreatment of the cells for 1 h with sphingomyelinase (0.5 U/ml: a treatment known to disrupt sphingomyelin, a component of lipid rafts) was without effect on either cholesterol content or taurine efflux (Fig. 4.9 A, B). Pre-incubation of the cells with filipin (1-5 µg/ml), which forms filipin-cholesterol complexes in membranes, also had no effect on either basal- or receptor-stimulated taurine efflux (data not shown).

## **Discussion**

Cholesterol availability has previously been reported to modulate the activity of several different ion channels (Bolotina et al., 1989; Jennings et al., 1999; Martens et al., 2000, 2001; Lockwich et al., 2000), in addition to that of VSOAC. In agreement with previous studies of non-neural cells, depletion of cholesterol from SH-SY5Y cells by pretreatment with CD under relatively mild

conditions (5 mM for 10 min), facilitated the basal release of taurine in response to hypotonicity. However, the more significant observation was that depletion of cholesterol resulted in a further potentiation of receptor-stimulated osmolyte efflux, such that, following CD pretreatment, a synergistic, rather than additive, release of taurine above controls was observed (Fig. 4.3). The ability of cholesterol depletion to enhance receptor-stimulated taurine efflux was observed for all three receptors examined (mAChR, PAR-1 and S1P), even though they operate via different signal transduction mechanisms. Although cholesterol depletion has been reported to alter ligand binding to cell-surface receptors (Burger et al., 2000), CD pretreatment of SH-SY5Y cells increased the efficacy of thrombin- or Oxo-M-mediated taurine release, but had no effect on agonist potency (Fig. 4.4). In addition, because cholesterol depletion had no effect on the extent of mAChR-stimulated phosphoinositide turnover (Fig. 4.8), this result argues against either non-specific effects of CD or increases in second messenger production as causes for enhancement of receptor-stimulated taurine release. Taken collectively, the results suggest that the ability of cholesterol depletion to potentiate receptor-stimulated osmolyte efflux occurs independently of any changes in either ligand binding or monitored signal transduction events and that the results are consistent with a modulatory role of the sterol either at the VSOAC channel itself, or alternatively, at an associated membrane domain.

Further evidence for a role of cholesterol in the regulation of receptor-stimulated taurine efflux was obtained from experiments in which CD, complexed with cholesterol, was used as a donor to restore cholesterol to CD-pretreated

cells. When this approach was employed, it was observed that the CD-mediated potentiation of both PAR-1 and mAChR-stimulated taurine release could be reversed when the cells were presented with a CD:cholesterol (1:8) complex (Fig. 4.7). Under these conditions, cellular cholesterol concentrations were increased by 65% and this was accompanied by a significant inhibition (51-70%) of both basal- and receptor-stimulated taurine efflux. Administration of epicholesterol, a chiral analog of cholesterol, was similarly able to reverse the stimulatory effects of cholesterol depletion on taurine release, and also reduced efflux to below control levels. Since administration of either cholesterol- or epicholesterol-CD complexes to SH-SY5Y cells did not result in any change in the extent of mAChR-stimulated phosphoinositide turnover, an effect of these sterols on signaling events appears unlikely. The more pronounced ability of cholesterol (than epicholesterol) to inhibit agonist-stimulated taurine efflux (61-77% vs. 25-29%) could reflect either a more limited rate of exchange of epicholesterol into the cells, or alternatively, differential abilities of cholesterol and epicholesterol to form sterol-protein interactions (Zidovetzki and Levitan, 2007). Elevated cholesterol concentrations have also been reported to inhibit basal VSOAC activity in endothelial and intestinal 407 cells (Levitan et al., 2000; Lim et al., 2006), but not in ascites cells (Klausen et al., 2006). These results indicate that the degree of cholesterol regulation of VSOAC activity may be cell-type specific.

One of the most salient features of volume-dependent osmolyte efflux monitored following receptor activation is that the threshold osmolarity for release

is significantly reduced, thereby allowing osmolyte release to occur at more physiologically relevant osmolarities (Mongin and Kimelberg, 2002; Heacock et al., 2004, 2006; Cheema et al., 2007). A major conclusion to emanate from the present study is that cholesterol depletion can further increase this ability of receptor activation to facilitate osmolyte efflux and moreover, that the effect is most marked under conditions of isotonicity or mild hypotonicity (295 mOsM: a 13% reduction in osmolarity, Fig. 4.6). From these results, we conclude that cholesterol depletion and receptor activation can act in concert to promote osmolyte efflux and, by inference, cell volume regulation under conditions of minimal reductions in osmolarity. A limited degree of facilitation of taurine efflux by thrombin in cholesterol-depleted cells was even detected under mildly hypertonic conditions (370 mOsM), but not under conditions of more severe hypertonicity, a result that indicates that removal of cholesterol permits the VSOAC channel to remain partially open under conditions of mild hypertonicity. In this context, Levitan et al., (2000) have proposed that cholesterol content alters the equilibrium between the closed and open states of the VSOAC, with cholesterol depletion favoring the existence of an open channel. Since VSOAC, via the release of glutamate, another organic osmolyte, has also been implicated in cell-cell signaling events under isotonic conditions (Mulligan and MacVicar, 2006), regulation of channel activity by cholesterol availability could be of physiological significance under conditions of both isotonicity and hypotonicity. Although cholesterol depletion also enhanced the basal release of taurine from SH-SY5Y cells in a volume-dependent manner, the stimulatory effect was less

marked than that observed for receptor activation and only reached significance at an osmolarity of 230 mOsM. In contrast to previous studies obtained for non-neural cells in which the stimulatory effects of cholesterol depletion on VSOAC activity were abrogated by larger reductions in osmolarity (Levitan et al., 2000; Klausen et al., 2006; Lim et al., 2006), cholesterol depletion of SH-SY5Y cells was observed to facilitate the basal efflux of taurine even under conditions of a 40% reduction in osmolarity. As observed for control cells, inclusion of DCPIB attenuated the increases in both basal- and receptor-stimulated efflux of taurine from CD-pretreated SH-SY5Y cells. Thus, the increased release of osmolytes from cholesterol-depleted cells is also mediated by a VSOAC.

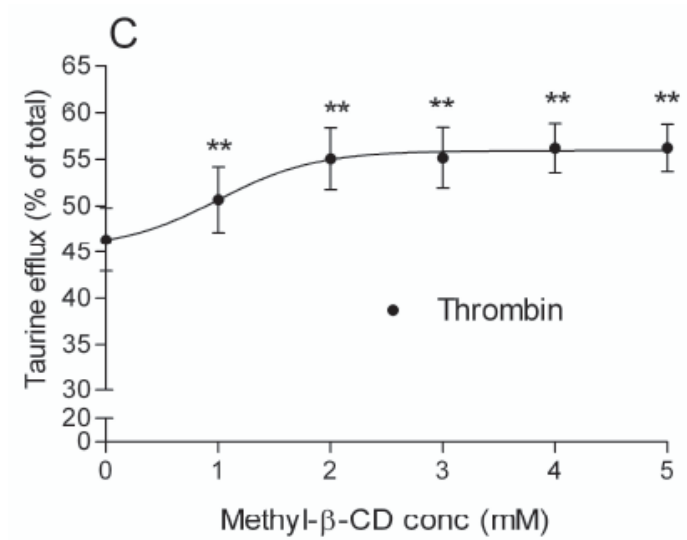
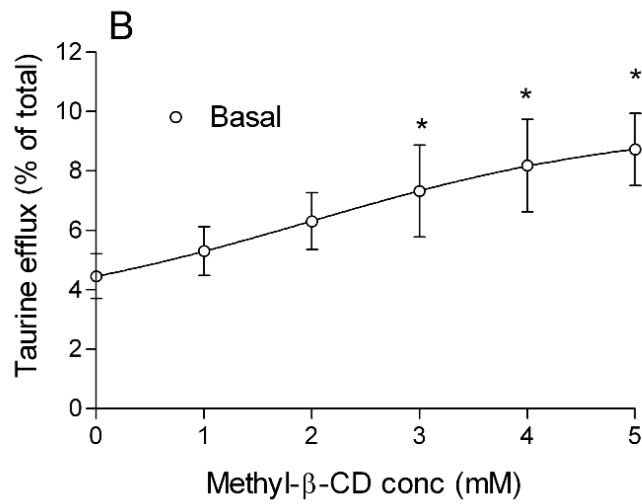
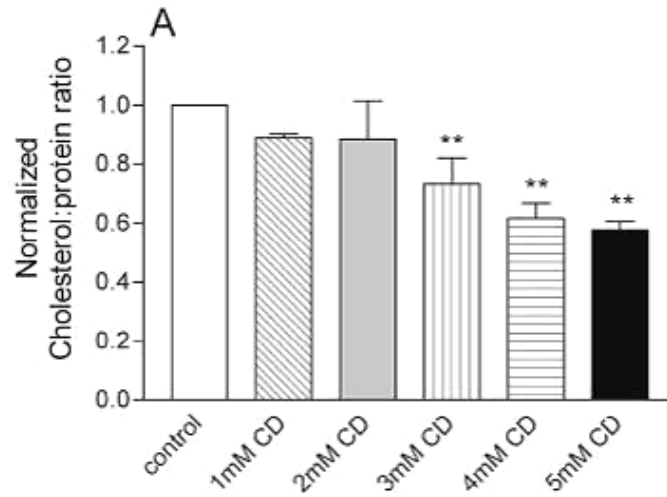
The functional relationship between alterations in cholesterol content and VSOAC activity remains uncertain. Two major roles for cholesterol in the regulation of VSOAC have previously been proposed, i.e. that the channel is localized to lipid rafts or caveolae (Trouet et al., 1999, 2000; Ullrich et al., 2006), or alternatively, that cholesterol-induced changes in the biophysical properties of the membrane result in an altered VSOAC activity (Levitan et al., 2000; Romanenko et al., 2004; Byfield et al., 2006). In SH-SY5Y cells, depletion of cholesterol with CD, a treatment documented to disrupt lipid microdomains (Brown and London, 2000; Zidovetzki and Levitan, 2007), resulted in an enhancement of both basal- and receptor-stimulated taurine efflux. Although one interpretation of this result is that the VSOAC channel is located in a lipid microdomain, under a negative constraint, this explanation seems unlikely since preincubation of SH-SY5Y cells with either cholesterol oxidase or

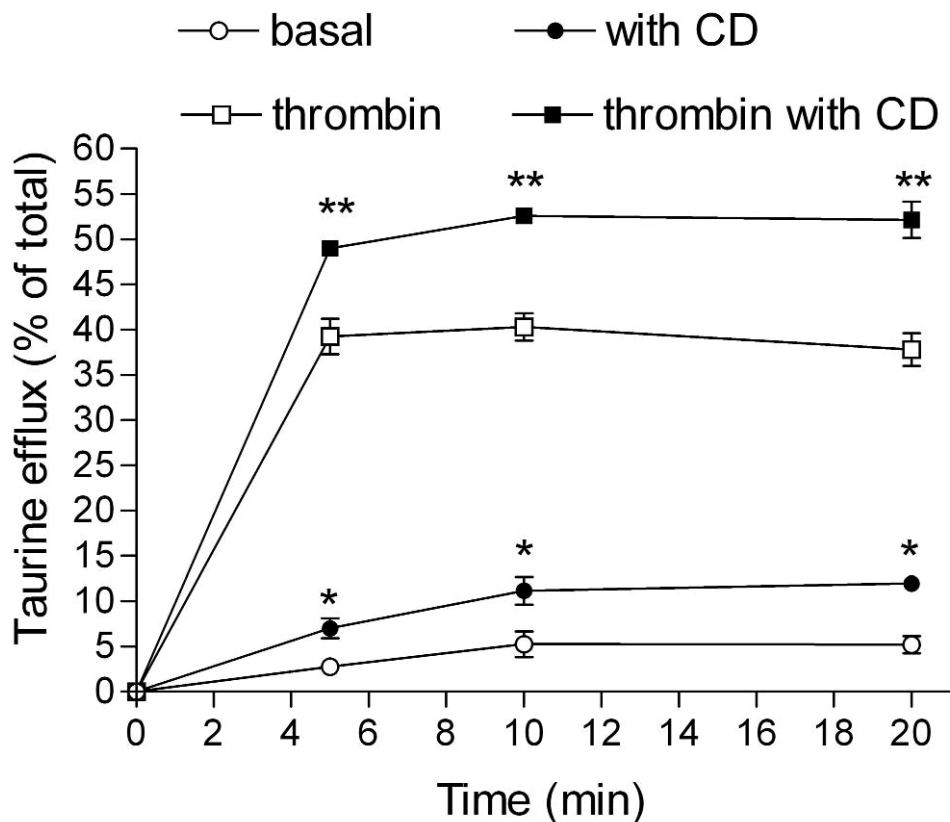


sphingomyelinase (or filipin), conditions under which lipid rafts are also disrupted (Samsonov et al., 2001), did not facilitate the release of osmolytes under either basal- or receptor-stimulated conditions (Fig. 4.9). Changes in the concentration of cell cholesterol are also documented to exert a major impact on the physical properties of the membrane lipid bilayer, with a reduction resulting in an increase in membrane fluidity, whereas elevated concentrations of cholesterol drive the fluid membrane bilayer into a more rigid state (Xu and London, 2000). In SH-SY5Y cells, the ability of cholesterol depletion to facilitate VSOAC activity, while cholesterol replenishment inhibits channel activity, is consistent with the possibility that VSOAC is regulated by changes in membrane fluidity. This conclusion is strengthened by the observation that epicholesterol, a chiral analog of cholesterol that exhibits similar properties as cholesterol in terms of its effect on bulk membrane fluidity (Gimpl et al., 1997), essentially mimicked the ability of cholesterol to reverse the stimulation of taurine efflux elicited by cholesterol depletion. In addition, although cholesterol oxidase treatment, like that of CD, resulted in a loss of ~50% of cell cholesterol, the oxidation product, namely 4-cholesten-3-one, exhibits the same membrane fluidity properties as cholesterol (Gimpl et al., 1997) and this may account for the absence of change in either the basal- or receptor-stimulated osmolyte release. The results obtained for SH-SY5Y cells are in agreement with those previously published for endothelial cells (Levitan et al., 2000; Romanenko et al., 2004; Byfield et al., 2006) and are consistent with a change in membrane fluidity, rather than a localization of the channel to a lipid microdomain, being a major determinant of VSOAC activity.

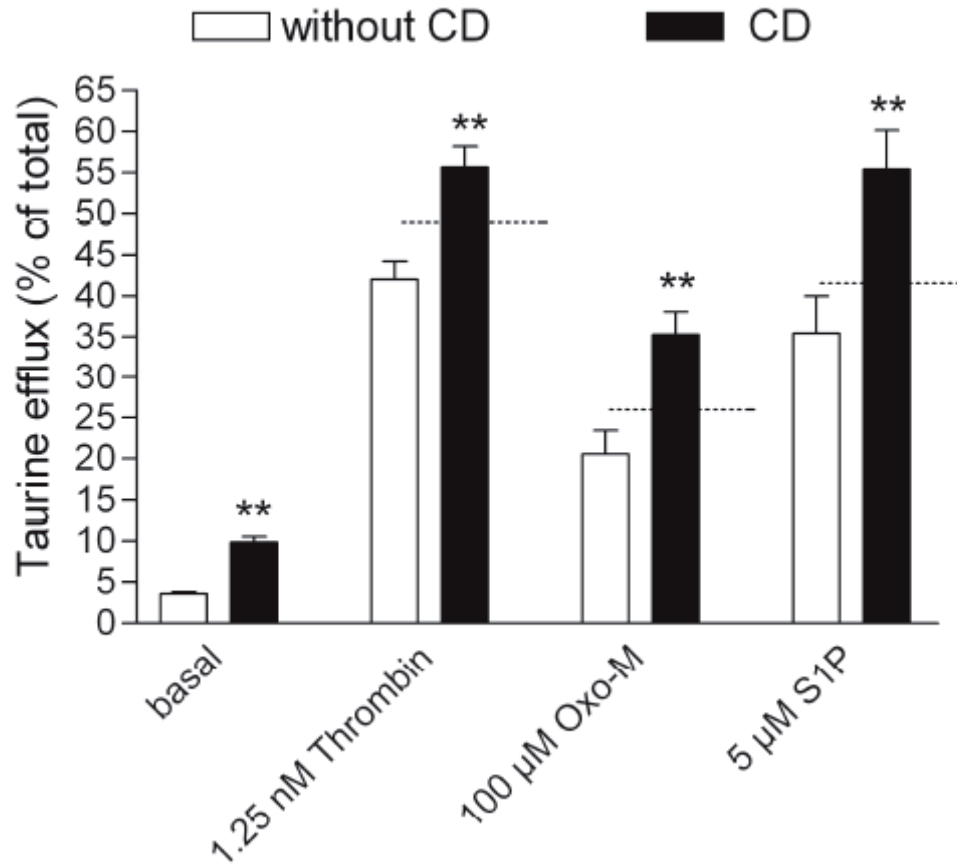
Alterations in cholesterol content within the central nervous system occur in conditions associated with genetic deficits in cholesterol synthesis/trafficking, such as Niemann-Pick type C, Smith-Lemli-Opitz syndrome or Tangier disease (Maxfield and Tabbas, 2005) and also following dietary modification (Foot et al., 1982) or administration of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (Zipp et al., 2007). Thus changes in cholesterol content *in vivo*, could serve an important modulatory role for VSOAC and other ion channels. In this context, it is relevant to note that VSOAC activity is markedly reduced in the fibroblasts of patients with Niemann-Pick's disease, a condition in which cholesterol content is elevated (Lim et al., 2006). The present results, obtained for SH-SY5Y neuroblastoma cells, are also consistent with the possibility that cholesterol availability, acting in concert with receptor activation, provides an additional means whereby volume regulation in neural cells is modulated.

**Figure 4.1. Cholesterol depletion enhances both basal- and thrombin-stimulated taurine efflux.** (A) SH-SY5Y neuroblastoma cells were pretreated for 10 min at 37°C with either serum-free media (control) or with increasing concentrations of CD (1 – 5 mM) dissolved in serum-free media. The cells were then washed twice with 2 ml of isotonic buffer A and cholesterol content measured by the Amplex Red assay (See Methods). Cholesterol values, calculated as µg/mg of protein, have been normalized to the value of cholesterol in control cells (1.0) to account for inter-experimental variation. \*\*, Different from control,  $p < 0.01$  (by ordinary ANOVA followed by Dunnett's multiple comparisons test). (B, C) Cells prelabeled with [<sup>3</sup>H]taurine were treated under similar conditions as in (A) and then incubated in 230 mOsM buffer A in the absence (Basal) or presence of 1.25 nM thrombin. Reactions were terminated after 5 min and release of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 3-7 independent experiments. \*, Different from basal control,  $p < 0.05$ ; \*\*, different from thrombin control,  $p < 0.01$  (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).



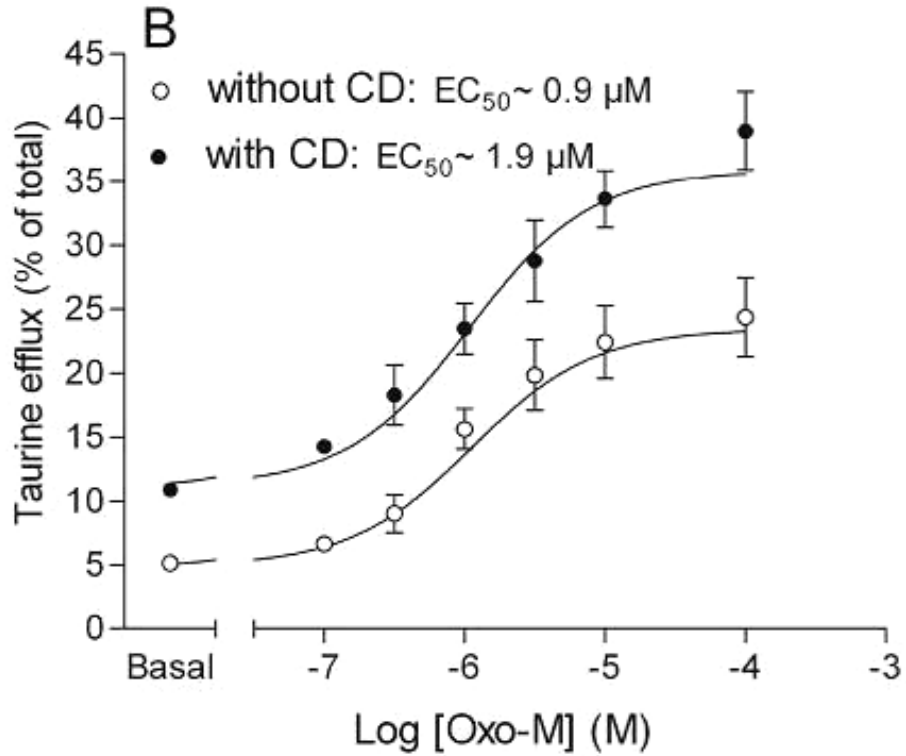
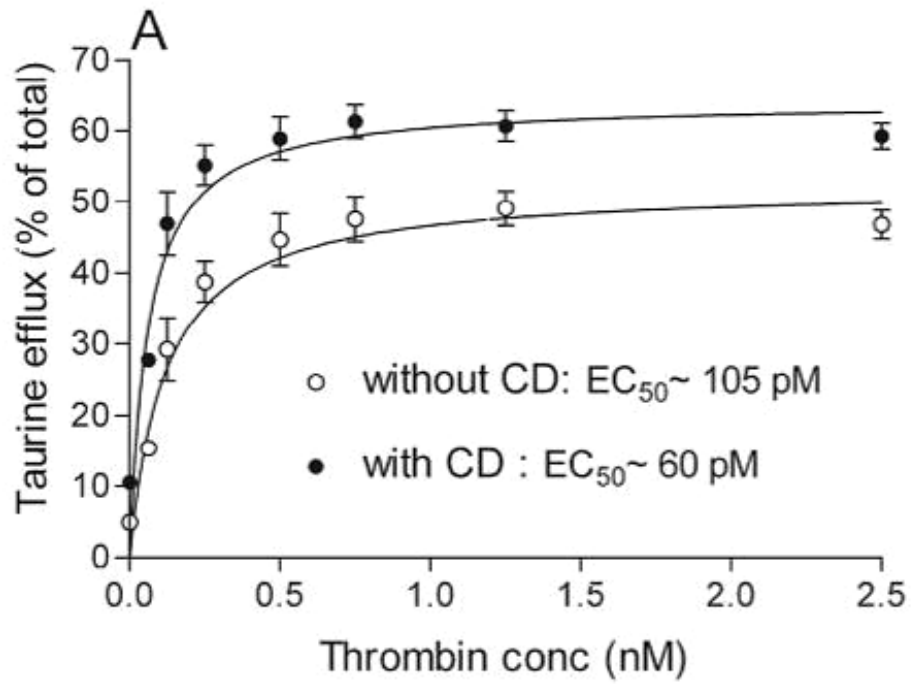


**Figure 4.2. Time-course of volume-dependent taurine efflux after cholesterol depletion.** SH-SY5Y neuroblastoma cells that had been prelabeled with [<sup>3</sup>H]taurine were pretreated for 10 min at 37°C with serum-free media or 5mM CD dissolved in serum-free media. The cells were then washed twice with 2 ml of isotonic buffer A and then incubated in 230 mOsM buffer A in the presence or absence of 1.25 nM thrombin. Reactions were terminated at the times indicated and taurine release measured. Results are expressed as basal taurine efflux (-○- without CD, -●- with CD) or thrombin-stimulated taurine efflux (-□- without CD, -■- with CD) (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 independent experiments performed in triplicate. \*, Different from basal efflux without CD, p<0.05; \*\*, different from thrombin-stimulated efflux without CD, p< 0.05 (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

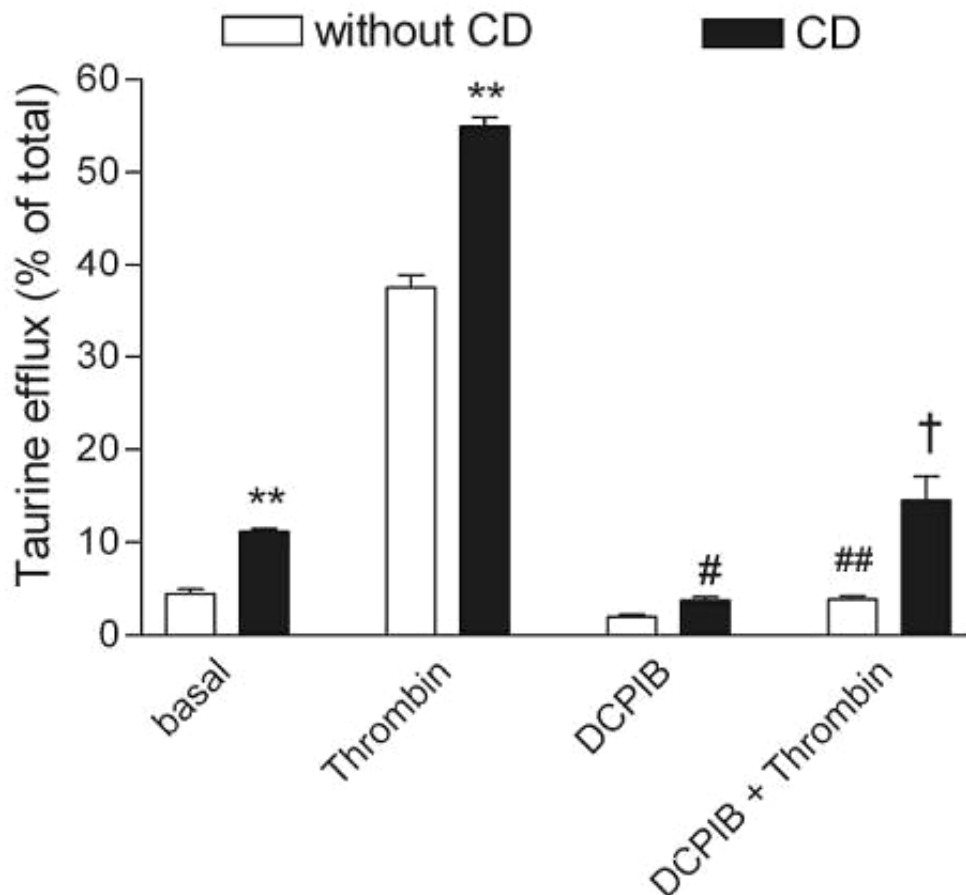


**Figure 4.3. Cholesterol depletion facilitates taurine efflux elicited by the activation of multiple receptors.** Cells pre-labeled with [<sup>3</sup>H]taurine were pretreated for 10 min at 37°C with either serum-free media or 5mM CD. The cells were then washed twice with 2 ml of isotonic buffer A and incubated in 230 mOsM buffer A for 5 min in the presence or absence of the receptor agonists at the concentrations indicated. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 3 - 4 independent experiments. \*, \*\*, Different from without CD, p< 0.05; p< 0.01 (by paired t-test and ordinary ANOVA followed by Dunnett's multiple comparisons test).

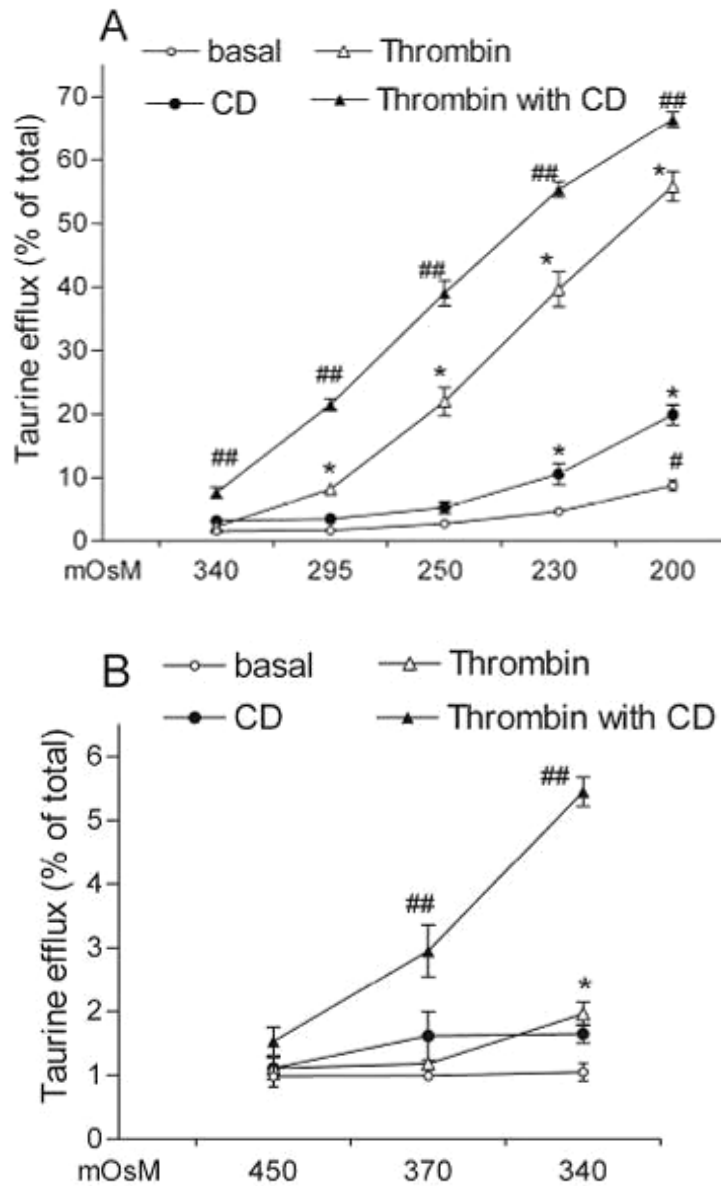
**Figure 4.4. CD pretreatment increases agonist efficacy for taurine efflux, but not agonist potency.** Cells prelabeled with [<sup>3</sup>H]taurine were pretreated for 10 min at 37°C with either serum-free media or 5 mM CD. The cells were then washed twice with 2 ml of isotonic buffer A and incubated in 230 mOsM buffer A in the presence or absence of (A) thrombin or (B) Oxo-M at the concentrations indicated. Reactions were terminated after 5 min and taurine efflux was monitored. Results are expressed as percentage of total soluble radioactivity released and are the means ± S.E.M. for 3-4 independent experiments. Where error bars are not shown, the values fell within the symbol. The calculated EC<sub>50</sub> values for stimulated taurine efflux for thrombin were 105 pM and 60 pM (in the absence and presence of CD, respectively) and 0.9 μM and 1.9 μM for Oxo-M (in the absence and presence of CD, respectively). At all concentrations of either thrombin or Oxo-M, taurine release was greater for the CD-pretreated cells than for control cells (p<0.05: by ordinary- [thrombin] or repeated measures [Oxo-M] ANOVA followed by Dunnett's multiple comparisons test).





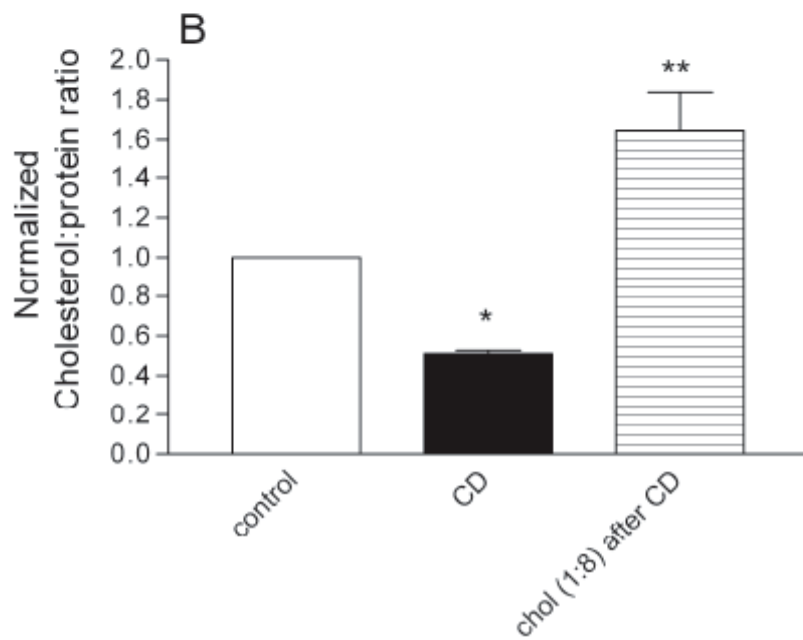
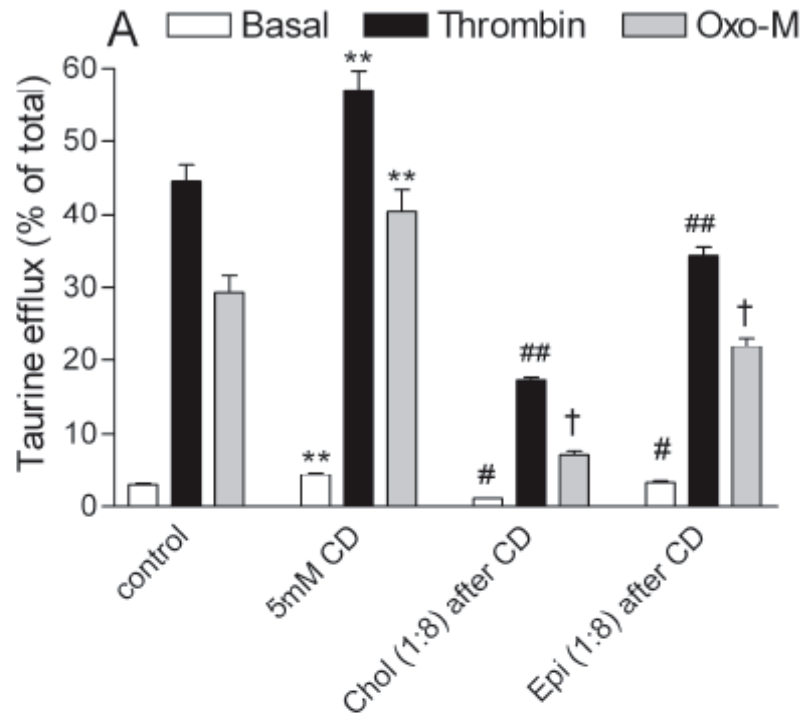


**Figure 4.5. Blockade of VSOAC with DCPIB attenuates the CD-induced facilitation of taurine release.** Cells prelabeled with [<sup>3</sup>H]taurine were pretreated with 20 μM DCPIB in either serum-free media or 5 mM CD for 10 min before incubation in hypotonic buffer A (230 mOsM) containing 20 μM DCPIB in the absence (open bars) or presence (black bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 independent experiments, each performed in triplicate. \*\*, Different from respective controls, p<0.01; # different from basal plus CD, p<0.01; ## different from thrombin alone, p<0.01; † different from thrombin plus CD, p< 0.01 (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).

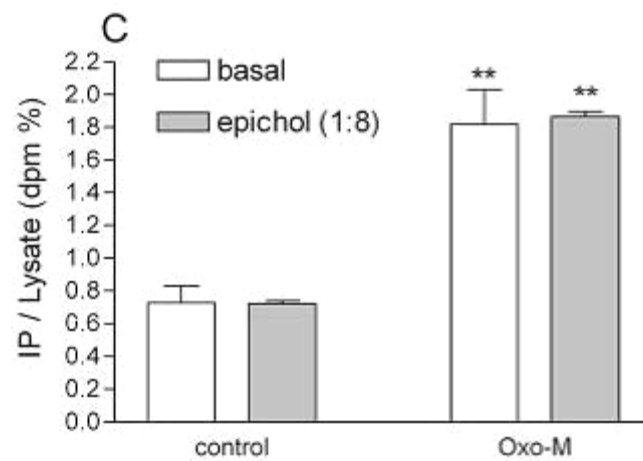
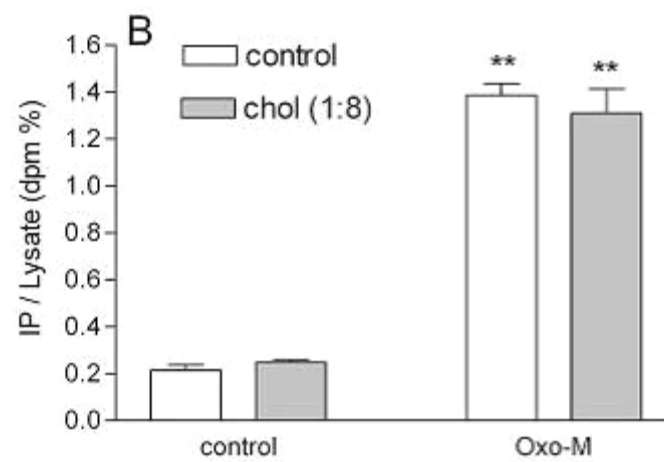
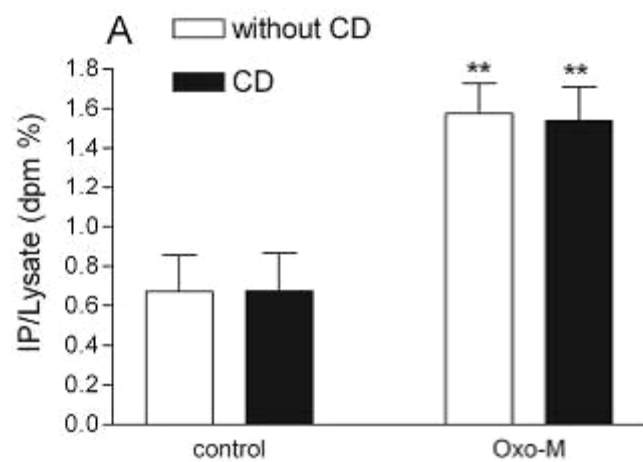


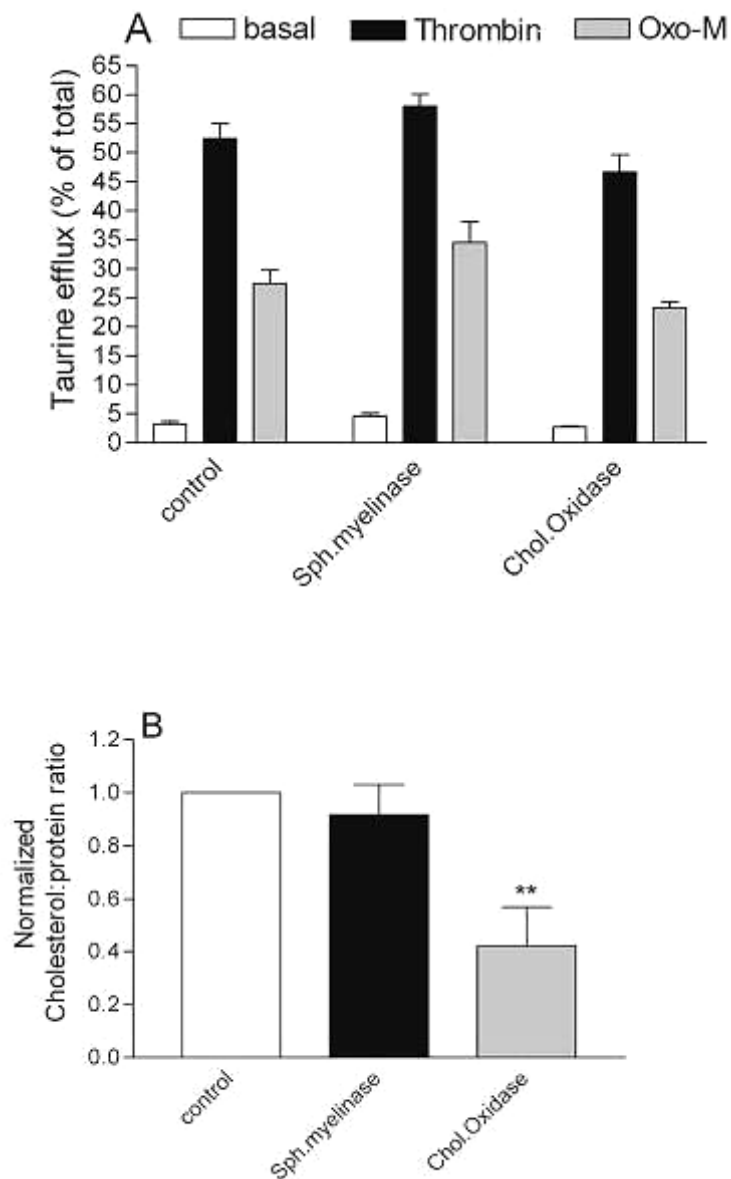
**Figure 4.6. Osmolarity dependence of basal- and thrombin-stimulated taurine efflux under control and cholesterol-depleted conditions.** Cells prelabeled with [<sup>3</sup>H]taurine were first pretreated for 10 min at 37°C with either serum-free media (open symbols) or 5 mM CD (closed symbols). The cells were then washed twice with 2 ml of isotonic buffer A and incubated in (A) hypo-osmolar buffers for 5 min or (B) hyperosmolar buffers for 10 min, at the osmolarities indicated in the absence (basal, ○, ●) or presence (Δ, ▲) of 1.25 nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 independent experiments, each performed in triplicate. \*, Different from basal, p < 0.05 (by repeated measures ANOVA followed by Dunnett's multiple comparison test); #, different from isotonic 340 mOsM, p < 0.01; ##, different from thrombin-stimulated efflux without CD, p < 0.05 (by ordinary ANOVA followed by Dunnett's multiple comparisons test).

**Figure 4.7. Enhancement of taurine efflux by CD is reversed by the re-addition of cholesterol or epicholesterol.** Cells that had been pre-labeled overnight with [<sup>3</sup>H]taurine were treated with either serum-free media or 5 mM CD for 10 min at 37°C. The cells were washed once with 2 ml of isotonic buffer A and incubated with serum-free media, cholesterol: CD (1:8 ratio) or epicholesterol:CD (1:8 ratio) for 1 h. The cells were washed once more with isotonic buffer A before incubation for 5 min with hypotonic buffer A (230 mOsM) in the absence (open bars) or presence of thrombin (1.25 nM; black bars) or Oxo-M (100 μM; grey bars). Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3-9 independent experiments, each performed in triplicate. \*\*, Different from control efflux in the absence of CD, p<0.01; #, different from basal efflux plus CD, p<0.05; ##, different from thrombin-stimulated efflux, p<0.05; † different from Oxo-M-stimulated efflux after CD, p< 0.01 (by ordinary ANOVA followed by Dunnett's multiple comparisons test). (B) Cells were treated the same way and cholesterol content measured by Amplex Red kit. Results (means ± S.E.M) are normalized as cholesterol: protein ratios from 4 independent experiments. \*, \*\*, Different from control cells, p< 0.05, p<0.01 (by repeated measures ANOVA followed by Dunnett's multiple comparisons test).



**Figure 4.8. Alterations in cholesterol content or replacement with epicholesterol do not modulate mAChR-stimulated phosphoinositide turnover.** Cells that had been prelabeled for 48 h with [<sup>3</sup>H]inositol were (A) pretreated with either serum-free media or 5 mM CD for 10 min or (B) pretreated with serum-free media or CD for 10 min followed by incubation with a cholesterol:CD complex (1:8) for 1 h or (C) pretreated with serum-free media or CD for 10 min followed by incubation with an epicholesterol:CD complex (1:8) for 1 h. The cells were then washed with isotonic buffer A and incubated for 10 min in hypotonic buffer A (230 mOsm) in the presence or absence of Oxo-M (100 μM). Reactions were terminated by the addition of trichloroacetic acid and the accumulation of radiolabeled inositol phosphates (IP) was monitored as an index of stimulated phosphoinositide turnover. Results are expressed as inositol phosphate (IP) release/total soluble radioactivity in cell lysates (IP/lysate; dpm %) and are the means ± S.E.M. for 3 - 4 independent experiments. \*\*, Different from respective controls, p < 0.001 (by ordinary ANOVA followed by Dunnett's multiple comparisons test).





**Figure 4.9. Pretreatment with cholesterol oxidase or sphingomyelinase has no effect on basal- or receptor-stimulated taurine efflux.** Cells that had been prelabeled overnight with [<sup>3</sup>H]taurine were treated with either serum-free media, 0.5 U/ml sphingomyelinase or 1 U/ml cholesterol oxidase for 1 h at 37°C. The cells were then washed twice with 2 ml of isotonic buffer A and incubated in hypotonic buffer A (230 mOsM) for 5 min in the absence (open bars) or presence of either thrombin (1.25 nM; black bars) or Oxo-M (100 μM; grey bars). Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 - 5 independent experiments. (B) Unlabeled cells were treated as above and cholesterol content measured by Amplex Red kit. Results (means ± S.E.M) are normalized as cholesterol: protein ratios from 3 independent experiments, each performed in triplicate. \*\*, Different from control cells, p<0.05 (by ordinary ANOVA followed by Dunnett's multiple comparisons test).

## References

- Abdullaev IF, Rudkouskaya A, Schools GP, Kimelberg HK and Mongin AA (2006) Pharmacological comparison of swelling-activated excitatory amino acid release and Cl<sup>-</sup> currents in cultured rat astrocytes. *J Physiol* 572:677-689.
- Allen JA, Halverson-Tamboli RA and Rasenick MM (2007) Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* 8:128-140.
- Bolotina V, Omelyanenko V, Heyes B, Ryan U and Bregestovski P (1989) Variations of membrane cholesterol alter the kinetics of Ca<sup>2+</sup>-dependent K<sup>+</sup> channels and membrane fluidity in vascular smooth muscle cells. *Pflugers Arch* 415:262-268.
- Brown DA and London E (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* 275:17221-17224.
- Burger K, Gimpl G and Fahrenholz F (2000) Regulation of receptor function by cholesterol. *Cell Mol Life Sci* 57:1577-1592.
- Byfield FJ, Hoffman BD, Romanenko VG, Fang Y, Crocker JC and Levitan I (2006) Evidence for the role of cell stiffness in modulation of volume-regulated anion channels. *Acta Physiol (Oxf)* 187:285-294.
- Cheema TA, Pettigrew VA and Fisher SK (2007) Receptor regulation of the volume-sensitive efflux of taurine and iodide from human SH-SY5Y neuroblastoma cells: differential requirements for Ca<sup>2+</sup> and protein kinase C. *J Pharmacol Exp Ther* 320:1068-1077.
- Cheema TA, Ward CE and Fisher SK (2005) Subnanomolar concentrations of thrombin enhance the volume-sensitive efflux of taurine from human 1321N1 astrocytoma cells. *J Pharmacol Exp Ther* 315:755-763.
- Christian AE, Haynes MP, Phillips MC and Rothblat GH (1997) Use of cyclodextrins for manipulating cellular cholesterol content. *J Lipid Res* 38:2264-2272.
- Foot M, Cruz TF and Clandinin MT (1982) Influence of dietary fat on the lipid composition of rat brain synaptosomal and microsomal membranes. *Biochem J* 208:631-640.
- Gimpl G, Burger K and Fahrenholz F (1997) Cholesterol as modulator of receptor function. *Biochemistry* 36:10959-10974.



- Heacock AM, Foster DJ and Fisher SK (2006) Prostanoid receptors regulate the volume-sensitive efflux of osmolytes from murine fibroblasts via a cyclic AMP-dependent mechanism. *J Pharmacol Exp Ther* 319:963-971.
- Heacock AM, Kerley D, Gurda GT, VanTroostenberghe AT and Fisher SK (2004) Potentiation of the osmosensitive release of taurine and D-aspartate from SH-SY5Y neuroblastoma cells after activation of M3 muscarinic cholinergic receptors. *J Pharmacol Exp Ther* 311:1097-1104.
- Jennings LJ, Xu QW, Firth TA, Nelson MT and Mawe GM (1999) Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle. *Am J Physiol* 277:G1017-1026.
- Klausen TK, Hougaard C, Hoffmann EK and Pedersen SF (2006) Cholesterol modulates the volume-regulated anion current in Ehrlich-Lette ascites cells via effects on Rho and F-actin. *Am J Physiol Cell Physiol* 291:C757-771.
- Lang F, Busch GL and Volkl H (1998) The diversity of volume regulatory mechanisms. *Cell Physiol Biochem* 8:1-45.
- Levitan I, Christian AE, Tulenko TN and Rothblat GH (2000) Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. *J Gen Physiol* 115:405-416.
- Lim CH, Schoonderwoerd K, Kleijer WJ, de Jonge HR and Tilly BC (2006) Regulation of the cell swelling-activated chloride conductance by cholesterol-rich membrane domains. *Acta Physiol (Oxf)* 187:295-303.
- Lockwich TP, Liu X, Singh BB, Jadlowiec J, Weiland S and Ambudkar IS (2000) Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J Biol Chem* 275:11934-11942.
- Loveday D, Heacock AM and Fisher SK (2003) Activation of muscarinic cholinergic receptors enhances the volume-sensitive efflux of myo-inositol from SH-SY5Y neuroblastoma cells. *J Neurochem* 87:476-486.
- Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD and Tamkun MM (2000) Differential targeting of Shaker-like potassium channels to lipid rafts. *J Biol Chem* 275:7443-7446.
- Martens JR, Sakamoto N, Sullivan SA, Grobaski TD and Tamkun MM (2001) Isoform-specific localization of voltage-gated K<sup>+</sup> channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. *J Biol Chem* 276:8409-8414.

- Maxfield FR and Tabas I (2005) Role of cholesterol and lipid organization in disease. *Nature* 438:612-621.
- Mongin AA and Kimelberg HK (2002) ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. *Am J Physiol Cell Physiol* 283:C569-578.
- Mongin AA and Kimelberg HK (2005) ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple  $\text{Ca}^{2+}$ -sensitive mechanisms. *Am J Physiol Cell Physiol* 288:C204-213.
- Mulligan SJ and MacVicar BA (2006) VRACs CARVe a path for novel mechanisms of communication in the CNS. *Sci STKE* 2006:pe42.
- Nilius B and Droogmans G (2003) Amazing chloride channels: an overview. *Acta Physiol Scand* 177:119-147.
- Pasantes-Morales H, Cardin V and Tuz K (2000) Signaling events during swelling and regulatory volume decrease. *Neurochem Res* 25:1301-1314.
- Ramos-Mandujano G, Vazquez-Juarez E, Hernandez-Benitez R and Pasantes-Morales H (2007) Thrombin potently enhances swelling-sensitive glutamate efflux from cultured astrocytes. *Glia* 55:917-925.
- Romanenko VG, Rothblat GH and Levitan I (2002) Modulation of endothelial inward-rectifier  $\text{K}^+$  current by optical isomers of cholesterol. *Biophys J* 83:3211-3222.
- Romanenko VG, Rothblat GH and Levitan I (2004) Sensitivity of volume-regulated anion current to cholesterol structural analogues. *J Gen Physiol* 123:77-87.
- Samsonov AV, Mihalyov I and Cohen FS (2001) Characterization of cholesterol-sphingomyelin domains and their dynamics in bilayer membranes. *Biophys J* 81:1486-1500.
- Thompson AK and Fisher SK (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J Pharmacol Exp Ther* 252:744-752.
- Trouet D, Hermans D, Droogmans G, Nilius B and Eggermont J (2001) Inhibition of volume-regulated anion channels by dominant-negative caveolin-1. *Biochem Biophys Res Commun* 284:461-465.

- Trouet D, Nilius B, Jacobs A, Remale C, Droogmans G and Eggermont J (1999) Caveolin-1 modulates the activity of the volume-regulated chloride channel. *J Physiol* 520 Pt 1:113-119.
- Ullrich N, Caplanusi A, Brone B, Hermans D, Lariviere E, Nilius B, Van Driessche W and Eggermont J (2006) Stimulation by caveolin-1 of the hypotonicity-induced release of taurine and ATP at basolateral, but not apical, membrane of Caco-2 cells. *Am J Physiol Cell Physiol* 290:C1287-1296.
- Xu X and London E (2000) The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry* 39:843-849.
- Zidovetzki R and Levitan I (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* 1768:1311-1324.
- Zipp F, Waiczies S, Aktas O, Neuhaus O, Hemmer B, Schraven B, Nitsch R and Hartung HP (2007) Impact of HMG-CoA reductase inhibition on brain pathology. *Trends Pharmacol Sci* 28:342-349.

## CHAPTER 5

### CONCLUSION

As the extent of cell swelling in the brain is constrained by the encasing skull, pathological conditions that disturb cell volume homeostasis can severely compromise neural function and survival. In an attempt to counteract the osmolarity disturbance and restore normal cell volume, neural cells initiate volume regulatory mechanisms by modifying the concentration of osmotically active osmolytes (Mulligan and MacVicar, 2006).

Although the general characteristics of swelling-induced osmolyte release have been extensively studied, only recently has evidence emerged to indicate that certain pharmacologically distinct receptors are able enhance the regulatory processes. While previous studies have demonstrated the ability of a number of receptors to modulate VSOAC activity and osmolyte efflux from non-neural cells, at the initiation of this thesis only two distinct receptor agonists (ATP and Oxo-M) had been extensively characterized to enhance the efflux of organic osmolytes from astrocytes or neuroblastoma cells. Studies reported in this dissertation highlight the potentially important ability of GPCRs to potentiate osmolyte efflux through the VSOAC in response to hypo-osmotic stress from neural cells. The discovery that thrombin, acting via its PAR-1 receptor, could potentiate the release of both inorganic  $\text{Cl}^-$  and the organic osmolyte, taurine, in response to

pathological (>30%) or physiological (5-10%) reductions in osmolarity adds to the now growing list of receptors known to modulate this response. One notable feature in the study using 1321N1 astrocytoma cells (Chapter 2) was that thrombin-stimulation was able to substantially enhance osmolyte release even under isotonic conditions (340 mOsM; based on the osmolarity of the cell growth media). This suggests that the VSOAC that primarily responds to reductions in osmolarity is partially open in astrocytoma cells and may contribute to neural cell communication between glia and neurons. This is important as previously speculated for primary cultures of astrocytes (Mongin and Kimelberg, 2002). ATP, via its purinergic receptors, is known to enhance glutamate release even under physiological reductions in osmolarity, implicating the role of VSOAC and osmolyte efflux in crosstalk between synapses and neuronal excitability (Mongin and Kimelberg, 2002; Mulligan and MacVicar, 2006). The observation that receptor-stimulation (PAR-1 and mAChR) can lower the threshold osmolarity or “set-point” for osmolyte efflux (Chapter 2 and 3), suggests that these receptors may be modulating the volume-sensing and regulatory processes such that the cells respond immediately to hypo-osmotic stress. Together these data suggest that the processes in vivo whereby cells regulate volume may be under neuro-humoral control and are more dynamic than previously considered.

Experiments detailed in this thesis suggest that sub-nanomolar concentrations of thrombin known to be neuroprotective (Vaughan et al., 1995; Striggow et al., 2000) play an important role in potentiating osmoregulatory mechanisms in the CNS. Recently a role for thrombin in osmoregulation gained

further support from the demonstration that thrombin causes a decrease in the expression of the water selective channel, AQP 4, through a protein kinase C-dependent mechanism in primary astrocytes (Tang et al., 2007). These studies, along with studies in AQP4 knockout mice (Manley et al., 2000; Papadopoulos et al., 2004), could collectively suggest that thrombin is involved in the physiological as well as the pathological process of cerebral edema. Further studies in PAR-1 knockout mice may provide a means for investigating thrombin signaling in the nervous system and its role in osmoregulation.

One feature common to all four receptors in SH-SY5Y neuroblastoma cells (mAChR, PAR-1, S1P and LPA) now implicated in regulating VSOAC activity, is that their activation leads to the release of intracellular stores of  $\text{Ca}^{2+}$  and PKC activation. However, no simple relationship exists between the magnitude of receptor-mediated increases in  $[\text{Ca}^{2+}]_i$  and the extent of osmolyte release. For example, the rank order of efficacy for  $\text{Ca}^{2+}$  mobilization (Oxo-M > LPA > thrombin > S1P) differs considerably from that of osmolyte release (thrombin > S1P > Oxo-M > LPA). One possibility is that each receptor is linked to changes in  $[\text{Ca}^{2+}]_i$  via an activation of phospholipase C. However, although thrombin enhances phosphoinositide turnover in 1321N1 astrocytoma, this is not the case in SH-SY5Y neuroblastoma cells, suggesting that another signaling mechanism for calcium mobilization is involved. Thus, although the mechanism for a rise in  $[\text{Ca}^{2+}]_i$  remains to be determined, the experiments indicate that osmolyte release can be triggered by  $\text{Ca}^{2+}$ -mobilizing receptors that operate via

PLC-coupled (e.g., mAChR) or PLC-independent mechanisms (e.g., thrombin, S1P and LPA).

Experiments in this thesis also provide evidence for the involvement of PKC activity in the modulation of receptor-mediated osmolyte release. Thrombin- and Oxo-M- stimulated taurine release share this property, as is evident from the ability of chelerythrine, a PKC inhibitor, to partially inhibit organic osmolyte efflux response. However an interesting observation in this thesis is the differential regulation of release of the inorganic osmolyte,  $\text{Cl}^-$ , from the organic osmolyte, taurine, by  $\text{Ca}^{2+}$  and PKC after PAR-1 or mAChR activation (Chapter 3). In contrast to receptor-stimulated taurine efflux that is significantly inhibited by removal of  $\text{Ca}^{2+}$  and PKC (Fig 3.8 and 3.11),  $\text{Cl}^-$  efflux ( $^{125}\text{I}^-$  used as a tracer) in response to receptor stimulation was much less affected. These results could have two implications. The first is that the two receptors could activate a signaling pathway upstream of  $\text{Ca}^{2+}$  or PKC that may preferentially regulate the release of  $\text{Cl}^-$  rather than the organic osmolytes. Alternatively, the presence of separate VSOAC channels or existence of different isoforms of the channel activated by the GPCRs could account for these results. This possibility has been previously suggested for cell lines such as hepatoma, HeLa, epithelial and mammary tissue (Stutzin et al., 1999; Shennan and Thomson, 2000; Junankar et al., 2002). In Chapter 3, the kinetic and pharmacological analysis of the release of the two osmolytes ( $\text{Cl}^-$  and taurine) suggests the mediation of a common channel, especially considering the data obtained with DCPIB, an inhibitor considered to be most specific, yet identified for swelling activated  $\text{Cl}^-$  current

(Abdullaev et al., 2006). However caution in interpretation is still warranted due to the lack of knowledge of the mechanism of inhibition of these blockers and the unknown molecular identity of VSOAC.

Results obtained in Chapter 4 represent a new line of investigation on the role of cholesterol in modulating VSOAC activity. Previous studies suggest that VSOAC can be regulated by cholesterol either by changes in the physical properties of the membrane (Romanenko et al., 2004), caveolae/lipid rafts (Ullrich et al., 2006), or recruitment by cholesterol containing vesicles (Lim et al., 2006). In this thesis, I provide evidence that cholesterol depletion further potentiates the receptor-stimulated taurine efflux in SH-SY5Y neuroblastoma cells, mainly due to an influence on the biophysical properties of the membrane. Cholesterol is one of the major lipid components of mammalian membranes, therefore, an increase in cholesterol content restricts the motion of phospholipids resulting in lipid ordering and decrease in membrane fluidity (Xu and London, 2000). Conversely, a reduction in cholesterol content increases membrane fluidity. My studies, which demonstrate a potentiation of the organic osmolyte efflux response with decreasing cholesterol content, and an inhibition of taurine release with an increased cholesterol content, are consistent with the proposal that changes in membrane fluidity regulate the ability of receptor activation to enhance osmolyte release. Although compartmentation of signaling proteins in lipid rafts or caveolae might provide a mechanism of regulation based on the proximity of VSOAC and second messengers, my data suggest that this is not the case in SH-SY5Y neuroblastoma cells since monitored cell signaling events,



such as phosphoinositide turnover, appeared to be unchanged by cholesterol depletion or repletion. Furthermore, my results show no change in PAR-1 or mAChR agonist potency by cholesterol depletion suggesting that the effects observed with cholesterol cannot be attributed to alterations at the receptor level, as previously observed for oxytocin, neurokinin and 5-hydroxytryptamine receptors (Burger et al., 2000).

Methyl- $\beta$ -Cyclodextrins (CDs) serve as important tools to remove or deliver cholesterol to cells. However it is important to note that the degree of cholesterol depletion may differ significantly between cell types even when comparable CD concentrations and exposure times are applied. Short (2-10 min) exposure and/or low mM concentrations are thought to preferentially remove cholesterol from lipid rafts (Zidovetski and Levitan, 2007). Although only a limited amount of information is available about the rate of transfer of sterols back into cells, substitution of cholesterol with its structural analogs is considered a strong tool to discriminate between specific and non-specific effects of cholesterol. Epicholesterol differs from cholesterol only in the rotational angle of hydroxyl group at position 3 and due to its similar biophysical properties on the membrane, this analog mimics the ability of cholesterol to reverse the enhancement induced by cholesterol depletion. However, the possibility of a change in the cellular distribution of cholesterol, sterol-protein interactions or accompanied cytoskeletal changes cannot be excluded as additional factors in the modulation of VSOAC function.

The central nervous system is particularly enriched in cholesterol. Alterations in cholesterol content occur not only in conditions associated with disorders of cholesterol metabolism or intracellular cholesterol transport, such as Niemann-Pick type C, Smith-Lemli-Opitz syndrome or Tangier disease (Maxfield and Tabbas, 2005) but also under conditions of dietary modification (Foot et al., 1982). My results raise the possibility that changes in cholesterol content could further serve an important modulatory role in the regulation of VSOAC under both isotonic and hypotonic conditions. In this context it should be noted that VSOAC activity is markedly reduced in fibroblasts of patients with Niemann-Pick C, a disease in which cholesterol content is increased (Lim et al., 2006).

Efforts to understand the mechanisms of regulatory volume decrease have yielded multiple factors that contribute to potentiating the osmolyte efflux response. This dissertation provides evidence that the activation of PAR-1 and mAChRs can enhance osmolyte efflux which is mediated through the VSOAC. An increased mobilization of  $\text{Ca}^{2+}$  and PKC activity are pre-requisites for the release of organic osmolyte but less so for the efflux of  $\text{Cl}^-$ , a result which suggests that the release of these two classes of osmolytes is differentially regulated. Furthermore, alteration in the membrane fluidity characteristics of the plasma membrane can further modulate the receptor-mediated release of osmolytes (Fig. 5.1). The novel role of PAR-1 in osmoregulation adds to the growing list of functions attributed to this GPCR in CNS physiology and pathology.

## **Relevance and Future Directions**

The relevance of these studies needs to be considered in terms of both therapeutic and pathological implications in the brain. Enhancement of VSOAC activity may not only potentiate the release of non-perturbing organic osmolyte such as taurine, but also the release of glutamate, which at high concentrations can be excitotoxic. However, the occurrence of swelling-activated channels in non-stimulated cells suggests a fundamental role for this current even under physiological conditions. Furthermore, since hypotonic cell swelling is accompanied by a reduction in active taurine re-uptake as well as regulation of  $K^+$  channels, attention also needs to be given to mechanisms that terminate the volume-regulatory response. Experiments in this dissertation outline mechanisms for response to acute hypotonic exposure, therefore studies investigating prolonged as well as gradual hypotonic exposure effects on the expression of enzymes, cellular signaling systems and volume-sensitive transporters need to be conducted.

Future studies also need to prove the correlation of the GPCR-stimulated osmolyte efflux to RVD by measurement of cell volume. Real time images of changes in cell volume may not only contribute to the understanding of the kinetics of volume correction but may also provide some evidence for the localization of volume shifts between dendritic processes and/or cell nucleus and the intracellular dynamics of this regulatory process.

In spite of more than a 30 year history on the study of cell volume regulatory mechanisms, the nature of the primary volume sensor as well as the

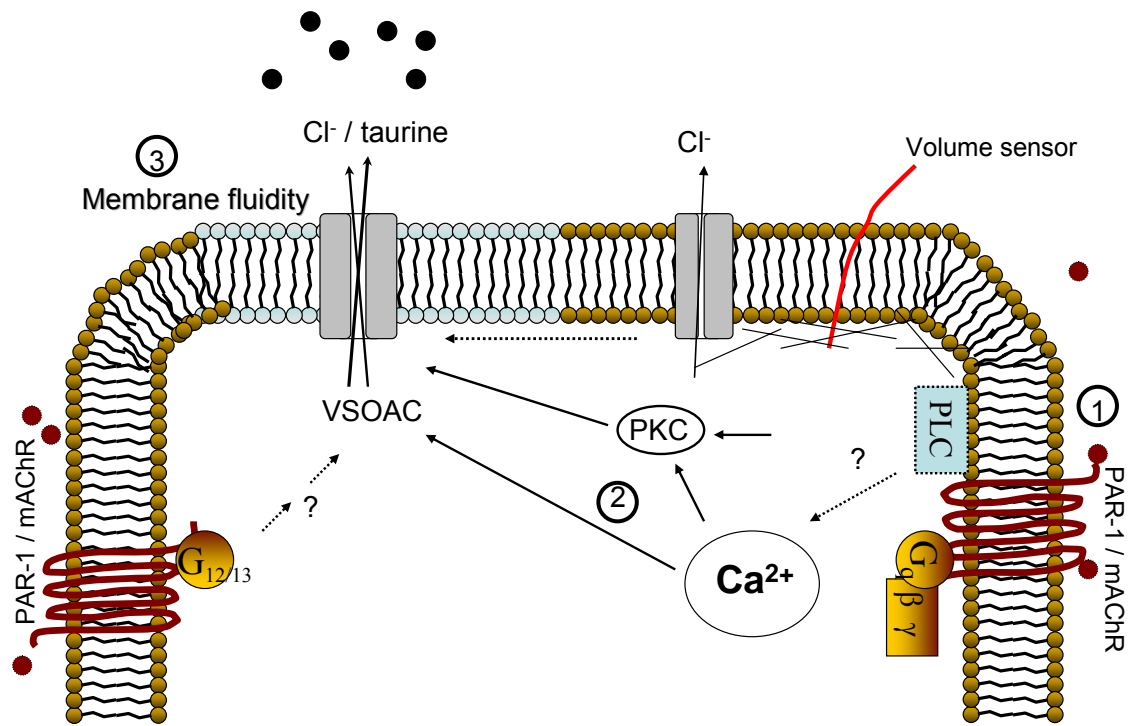
identity of VSOAC remain elusive. Each of the candidates proposed for the role has its pros and cons. Although it is a universal and evolutionary ancient structure, variations in volume-sensitive pathways in different cell types has made progress difficult in this field. Solving whether or not this diversity in the properties of the osmo-dependent anion fluxes reflects the heterogeneity of volume-sensitive anion channels in mammalian cells will require the molecular identification of the channel(s) involved.

To accelerate the progress in the field of osmoregulation it seems critical to also limit studies to well-characterized established cell models with concurrent measurement of Cl<sup>-</sup> current by patch-clamp, organic osmolyte efflux and volume change. Important information may also be obtained using molecular biological approaches in genetically characterized species such as *Caenorhabditis elegans* and/or zebrafish (Denton et al., 2005; Yin et al., 2007). Useful information and insights may be obtained by experimental techniques such as microarray or proteomics. It will also be important to carry out definitive experiments directed at establishing whether CIC proteins are candidates for VSOAC. This issue is all the more important as several proteins have been assigned as the swelling-activated Cl<sup>-</sup> channel, resulting in confusion in the Cl<sup>-</sup> channel field for many years (Jentsch et al., 2002).

In addition, it should be noted that although taurine can have agonist properties at both GABA<sub>A</sub> and glycine receptors (Hussy et al., 1997), the role(s) of taurine in the brain, besides serving as a volume regulatory osmolyte, is still not clear. However, there is an emerging body of data suggesting the importance

of organic osmolytes in protein folding and stability (Ignatova and Gierasch, 2006; Lambert and Draper, 2007) cardiomyopathy and developmental disorders (Warskulat et al., 2007). The swelling activated  $\text{Cl}^-$  channels have also been suggested to play a role in tumor cell migration and cell cycle progression (Olsen et al., 2003), apoptosis (Okada et al., 2006), spermatozoa maturation and fertility (Yeung et al., 2006) and aqueous humour secretion (Do and Civan, 2006). Changes in cell volume also occur upon ischemic stroke, hypoxic insults, diabetic neuropathy and spreading depression which further emphasize the importance of studying swelling-activated  $\text{Cl}^-$  and organic osmolyte release pathways (Hoffman and Dunham, 1995; Kimelberg et al., 2006). The discovery that sub-nanomolar concentrations of thrombin can regulate osmolyte efflux from neural cells also lays ground for future experiments to study the role of thrombin in reducing brain swelling after intra-cerebral hemorrhage and injury.

Understanding the issues regarding the physiological, pathophysiological and pharmacological factors that either impair, or enhance volume regulatory processes, can help in the design of therapies that alleviate the neurological manifestations accompanying disorders of body fluid osmolarity in humans.



**Figure 5.1. Schematic of mechanisms regulating osmolyte efflux from neural cells.** In this dissertation (1) PAR-1 and mAChR receptors potentiate inorganic and organic osmolyte efflux through the VSOAC. (2) A rise in Ca<sup>2+</sup> and PKC activity are pre-requisites for this receptor-stimulated osmolyte efflux response and can differentially regulate the two osmolytes. (3) Membrane fluidity further enhances receptor-stimulated osmolyte release. Dotted lines represent signaling pathways implicated but not proven in this thesis.

## References

- Abdullaev IF, Rudkouskaya A, Schools GP, Kimelberg HK and Mongin AA (2006) Pharmacological comparison of swelling-activated excitatory amino acid release and Cl<sup>-</sup> currents in cultured rat astrocytes. *J Physiol* 572:677-689.
- Burger K, Gimpl G and Fahrenholz F (2000) Regulation of receptor function by cholesterol. *Cell Mol Life Sci* 57:1577-1592.
- Denton J, Nehrke K, Yin X, Morrison R and Strange K (2005) GCK-3, a newly identified Ste20 kinase, binds to and regulates the activity of a cell cycle-dependent ClC anion channel. *J Gen Physiol* 125:113-125.
- Do CW and Civan MM (2006) Swelling-activated chloride channels in aqueous humour formation: on the one side and the other. *Acta Physiol (Oxf)* 187:345-352.
- Foot M, Cruz TF and Clandinin MT (1982) Influence of dietary fat on the lipid composition of rat brain synaptosomal and microsomal membranes. *Biochem J* 208:631-640.
- Hoffmann EK and Dunham PB (1995) Membrane mechanisms and intracellular signalling in cell volume regulation. *Int Rev Cytol* 161:173-262.
- Hussy N, Deleuze C, Pantaloni A, Desarmenien MG and Moos F (1997) Agonist action of taurine on glycine receptors in rat supraoptic magnocellular neurones: possible role in osmoregulation. *J Physiol* 502 (Pt 3):609-621.
- Ignatova Z and Gierasch LM (2006) Inhibition of protein aggregation in vitro and in vivo by a natural osmoprotectant. *Proc Natl Acad Sci U S A* 103:13357-13361.
- Jentsch TJ, Stein V, Weinreich F and Zdebik AA (2002) Molecular structure and physiological function of chloride channels. *Physiol Rev* 82:503-568.
- Junankar PR, Karjalainen A and Kirk K (2002) The role of P2Y1 purinergic receptors and cytosolic Ca<sup>2+</sup> in hypotonically activated osmolyte efflux from a rat hepatoma cell line. *J Biol Chem* 277:40324-40334.
- Kimelberg HK, Macvicar BA and Sontheimer H (2006) Anion channels in astrocytes: biophysics, pharmacology, and function. *Glia* 54:747-757.
- Lambert D and Draper DE (2007) Effects of osmolytes on RNA secondary and tertiary structure stabilities and RNA-Mg<sup>2+</sup> interactions. *J Mol Biol* 370:993-1005.

- Lim CH, Schoonderwoerd K, Kleijer WJ, de Jonge HR and Tilly BC (2006) Regulation of the cell swelling-activated chloride conductance by cholesterol-rich membrane domains. *Acta Physiol (Oxf)* 187:295-303.
- Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P and Verkman AS (2000) Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat Med* 6:159-163.
- Maxfield FR and Tabas I (2005) Role of cholesterol and lipid organization in disease. *Nature* 438:612-621.
- Mongin AA and Kimelberg HK (2002) ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. *Am J Physiol Cell Physiol* 283:C569-578.
- Mulligan SJ and MacVicar BA (2006) VRACs CARVe a path for novel mechanisms of communication in the CNS. *Sci STKE* 2006:pe42.
- Okada Y, Shimizu T, Maeno E, Tanabe S, Wang X and Takahashi N (2006) Volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. *J Membr Biol* 209:21-29.
- Olsen ML, Schade S, Lyons SA, Amaral MD and Sontheimer H (2003) Expression of voltage-gated chloride channels in human glioma cells. *J Neurosci* 23:5572-5582.
- Papadopoulos MC, Saadoun S, Binder DK, Manley GT, Krishna S and Verkman AS (2004) Molecular mechanisms of brain tumor edema. *Neuroscience* 129:1011-1020.
- Romanenko VG, Rothblat GH and Levitan I (2004) Sensitivity of volume-regulated anion current to cholesterol structural analogues. *J Gen Physiol* 123:77-87.
- Shennan DB and Thomson J (2000) Further evidence for the existence of a volume-activated taurine efflux pathway in rat mammary tissue independent from volume-sensitive Cl<sup>-</sup> channels. *Acta Physiol Scand* 168:295-299.
- Strigrow F, Riek M, Breder J, Henrich-Noack P, Reymann KG and Reiser G (2000) The protease thrombin is an endogenous mediator of hippocampal neuroprotection against ischemia at low concentrations but causes degeneration at high concentrations. *Proc Natl Acad Sci U S A* 97:2264-2269.
- Stutzin A, Torres R, Oporto M, Pacheco P, Eguiguren AL, Cid LP and Sepulveda FV (1999) Separate taurine and chloride efflux pathways activated during regulatory volume decrease. *Am J Physiol* 277:C392-402.



- Tang Y, Cai D and Chen Y (2007) Thrombin inhibits aquaporin 4 expression through protein kinase C-dependent pathway in cultured astrocytes. *J Mol Neurosci* 31:83-93.
- Ullrich N, Caplanusi A, Brone B, Hermans D, Lariviere E, Nilius B, Van Driessche W and Eggermont J (2006) Stimulation by caveolin-1 of the hypotonicity-induced release of taurine and ATP at basolateral, but not apical, membrane of Caco-2 cells. *Am J Physiol Cell Physiol* 290:C1287-1296.
- Vaughan PJ, Pike CJ, Cotman CW and Cunningham DD (1995) Thrombin receptor activation protects neurons and astrocytes from cell death produced by environmental insults. *J Neurosci* 15:5389-5401.
- Warskulat U, Borsch E, Reinehr R, Heller-Stilb B, Roth C, Witt M and Haussinger D (2007) Taurine deficiency and apoptosis: findings from the taurine transporter knockout mouse. *Arch Biochem Biophys* 462:202-209.
- Xu X and London E (2000) The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry* 39:843-849.
- Yeung CH, Barfield JP and Cooper TG (2006) Physiological volume regulation by spermatozoa. *Mol Cell Endocrinol* 250:98-105.
- Yin X, Denton J, Yan X and Strange K (2007) Characterization of a novel voltage-dependent outwardly rectifying anion current in *Caenorhabditis elegans* oocytes. *Am J Physiol Cell Physiol* 292:C269-277.
- Zidovetzki R and Levitan I (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* 1768:1311-1324.
- Zipp F, Waiczies S, Aktas O, Neuhaus O, Hemmer B, Schraven B, Nitsch R and Hartung HP (2007) Impact of HMG-CoA reductase inhibition on brain pathology. *Trends Pharmacol Sci* 28:342-349.